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FACTORS INVOLVED IN THE GROWTH AND THE PYCNIDIUM FORMATION OF PLENODOMUS FUSCOMACULANS.[1]

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INTRODUCTION

The experimentation reported in this paper was begun at the botanical laboratory of the University of Michigan in 1913, continued at the Michigan Agricultural College during the next year, and finally completed in 1915 at the University laboratory.

The fungus *Plenodomus fuscomaculans* was obtained from badly cankered limbs of the apple (*Malus* spp.) which were sent to the Agricultural College laboratory in March, 1911, from Boyne City, Mich. Examination of the cankers at the time of receipt and field studies during the same month showed that the trouble was different from any of the described apple diseases. The cankers showed constant association with a pycnidium-forming fungus. This organism was obtained in pure culture from a single spore, and the causal relation of the fungus to the canker was proved by repeated inoculations and reisolations. A study of the organism, both on the host and in pure culture, showed that it was a Phoma-like member of the large group Sphaeropsidales, and it corresponded to the species described by Saccardo as *Aposphaeria fuscomaculans*.

The pycnidia, however, show morphological characters by which it is possible to segregate this fungus from the larger, poorly defined genus. These characters, which may be found in the material from the host, become very pronounced in culture. The pycnidia are more or less irregular in shape. The fruiting layer is usually folded so that the chamber is recessed instead of being smooth and regular. The pycnidia are beaked. The wall is composed of two distinct layers and is complete, even at the basal portion. It seems proper to emphasize the morphological character of the wall. Accordingly, the removal of this species from the genus Aposphaeria Berk., and the placing of it in the genus Plenodomus Preuss,

[1] Published with the permission of the Director of the Michigan Agricultural Experiment Station.

is proposed. The name of the fungus becomes, under this arrangement, *Plenodomus fuscomaculans* (Sacc.), n. comb.[1]

The present paper deals wholly with the physiological phase of my investigations, the phytopathological studies being reserved for another paper.[2]

The problem consisted of the investigation of the relations of the organism to the environment and the fitting of the environment to the organism—a marked reversal of the common practices in culture making.

HISTORICAL REVIEW

The history of the cultivation of micro-organisms is linked with the history of bacteriology and mycology. Progress in these sciences has been largely due to the clarifying effect of pure-culture methods. These originated with the discovery of the method by which media could be sterilized. It is a significant fact, and one which can be traced to the influence of these early experiments, that the solutions and materials used in the first crude cultures were the highly concentrated vegetable and animal decoctions and infusions which experience had shown to be highly liable to putrefaction. Mycology made great advance when, utilizing the newly discovered methods of isolation, the various groups of organisms were brought into pure culture by such masters as Brefeld, De Bary, Hansen, and Zopf. The earlier methods are in vogue to-day in the great bulk of mycological or applied work. In the cultural work of these pioneer studies nutrition was the only factor to which consistent attention was given.

The influence of other factors than nutrition was recognized early, but the methods of culture were varied but little to fit these conditions. Pasteur (1861)[3] showed the difference between aerobiosis and anaerobiosis, but this distinction long remained obscured by the problems of fermentation. The oxygen relations of fungi have been neglected in the ordinary cultural technique, since most fungi tolerate the conditions of the plugged flask or test tube. The sharp temperature requirements of some animal pathogens focused attention upon this factor very early, and accordingly incubators and devices to furnish constant temperature were developed. But there has been wide neglect of this factor. That bacteria grow best in a medium slightly alkaline to litmus and fungi in a medium slightly acid and that this difference can be used to advantage in isolation early became dicta of the sciences. The growth of organisms takes place within such wide limits in composition of culture media and

[1] A discussion of the morphology of this fungus was prepared for the 1915 Report of the Michigan Academy of Science. Delay in publishing this report makes it necessary to give the proposed change in nomenclature in this connection, with only a summary of the reasons for making the change. The latter publication may be looked to for a more complete account of the morphology of the fungus.

[2] The physiological work was suggested by Dr. C. H. Kauffman, of the University of Michigan, and has been done under his direction. I am also indebted to Dr. E. A. Bessey, of the Michigan Agricultural College, for advice and help throughout the investigation.

[3] Bibliographic citations in parentheses refer to "Literature cited," p. 766–769.

under such a range of conditions that accordingly these environmental factors have been neglected in culture work.

The emphasis placed upon nutrition has developed a great body of facts regarding media in which organisms will grow and rules for the preparation of the media. These compositions have the common characteristic that for the most part they present highly concentrated food supplies so complex as to defy analysis. The list includes beef infusion, prune juice, wort, Nähr solution, bread (plain or soaked in sugar solutions), vegetables of all kinds, and the long list of nutrient hydrogels. These media have given excellent vegetative growth; but if the common molds are excluded, it may be said that on the majority of media fructification is the exception rather than the rule.

In recent years many kinds of fruits, vegetables, and other biological products have been tried, either directly or as a base for a nutrient hydrogel. Some of these have produced fructification in forms which had previously grown only vegetatively in culture. Notable examples are corn meal, or corn-meal agar, which in the hands of Shear (Shear and Wood, 1913) and others led to an unraveling of the Gloeosporium complex, and oat agar, which in the hands of Clinton (1911) solved the historic *Phytophthora infestans* difficulty.

The complexity of the vast majority of combinations used in contemporary research, however, does not permit the analysis of the contributing factors which lead to fructification. The net contribution, therefore, toward a final analysis, which would furnish a key for unlocking closed approaches with other organisms is small, and further advance, so far as indicated by such work, must be by the same wasteful method of haphazard trial. It is known that organisms will grow under a vast assortment of conditions, but very little is known of the conditions which call out any particular phase of development.

Our knowledge of the physiology of micro-organisms has largely come from a study of their behavior under controlled conditions. The very analytical nature of the type of research used in the study of metabolism has made its methods in sharp contrast with those just described and has made possible evaluation of the various factors involved. The pure-culture methods just discussed and researches on the metabolism of micro-organisms have progressed side by side, and only slightly have the basic principles of the latter been influential in determining the course of the former. The art of cultivating organisms has indeed been developed, but this work is almost wholly empiric; although there is a mass of fundamental facts dealing with metabolism and with the reactions of plants to their environment, these for the most part are totally ignored in ordinary culture methods (Benecke, 1904; Behrens, 1904, p. 436–466).

Studies of the effects of various factors upon the metabolism of fungi naturally were made first with the nutrition of the micro-organisms. It was essential that the work be done with synthetic media; and along

with the development of the various synthetic culture solutions our knowledge of the nutritional requirements of micro-organisms has arisen (Pasteur, 1858, Raulin, 1869, Nägeli, 1880).

The gradual extension of the point of view of physiological response may be considered a guiding principle in cultivating organisms, and after a period of more or less accidental or random application of specific environments to influence growth or reproduction, a definite method based upon this teaching has been developed. Roux and Linossier (1890), with the animal pathogen, *Dematium albicans*, secured marked reactions to specific environmental factors, especially nutrition and oxygen. At the same time Winogradsky (1891) began his well-known work with the nitrifying organisms which he isolated by his method of "elective culture." This method, which consists essentially of so establishing the environment that only organisms of the desired type are able to develop, was carried to great perfection by Beijerinck (1901) with his similar "intensification" method. The bacteria and algæ with which Beijerinck worked required or tolerated different amounts of free oxygen, different nutrition, especially mineral salts, and different temperatures. Beijerinck used these differences as a means of isolation of various forms from a complex substratum (Stockhausen, 1907).

About the same time Klebs began his work on algæ and fungi in pure culture. Where others were concerned with growth, Klebs (1896) made the pure culture answer unsolved questions of life history. He (1913) recognized in the organism definite potentialities—the heredity of the organism. The manifestations of these potentialities are seen in the reactions to environment and in the limits of the various factors tolerated. The particular line of development followed by the organism can be traced to conditions outside of the potentiality, either inner conditions inaugurated by the environmental complex or outer conditions which work through their ability to set up certain internal effects. From this line of reasoning it was but a step to the position that the development of an organism is the resultant of the environment working upon definite internal potentialities of the organism and that with a given potentiality the same external conditions call forth the same response with the constancy of a chemical reaction. This response may be predicted from the type of conditions given, and in this regard Klebs (1900) announced the following propositions, as based upon his work:[1]

1. Growth and reproduction are life processes, which among all organisms depend upon different conditions; among the lower organisms, probably external conditions determine whether growth or reproduction ensue.

2. As long as the characteristic outer conditions for the growth of the lower organisms are present, reproduction does not set in. The favoring conditions for this process are always more or less unfavorable to growth.

[1] Author's translation.

3. Growth and reproduction differ also in that the working limits of the general life conditions, temperature, oxygen, etc., are narrower for reproduction than for growth. On this account growth can still take place, even if reproduction be limited through too weak or too strong influence of some factor.

4. Growth appears mostly as a preliminary for the initiation of reproduction, and, therefore, as an inner condition for it. Up to a certain limit, not directly growth, but the longer assimilation period is determinative.

From this point of view all the factors which influence life may be considered, and from the basis of the knowledge of their effects on growth, the ultimate effects of these factors upon reproduction may be predicted more or less accurately. This Klebs (1900, 1904) has done in the summary of his contributions to the physiology of reproduction.

Since that time research along this line may be divided into two types of endeavor: (1) Extending the groups to which the laws may be shown to apply and (2) the critical testing of the conclusions with the very organisms with which Klebs worked. The former has extended the limits so that none of the great groups of fungi or algæ are without many examples of the application of the conclusions. The work of the second type has opened up new points of view. Klebs in his experiments used a single strain, and the common experience, in repeating his experiments, is failure until the limits and life relations of the particular strain at hand are known. Accordingly, Kauffman (1908) has emphasized this point in his work with the same species of Saprolegnia that Klebs used; but where Klebs worked with one strain, Kauffman used two additional ones; and with this number of forms, each an entity and each varying from the other, Kauffman was able to show that within the limits of each the conclusions were valid. This work emphasizes a point which Klebs has made for his various forms, that each is a specific potentiality, but it makes the specific potentialities innumerable in their scope.

The particular organism with which I worked was one closely related to the large genus Phoma. This group, although containing many species, some of great economic importance, had received little attention from a physiological point of view. There have been no attempts to test the validity of Klebs's conclusions for the Sphaeropsidales.

Ternetz (1907) isolated from the roots of species of Vaccinium and Oxycoccus a series of *Phoma* spp. suspected of being mycorrhiza-producing forms. These organisms were grown in pure culture on synthetic media, and their relations to oxygen, nitrogen, and mineral salts were determined with great care. They were found to be sensitive to a restriction of the oxygen supply, especially when growing in a medium poor in nitrogen. These organisms were shown to have the power of utilizing nitrogen from the air. Saida (1902) has claimed the same for *Phoma betae*.

Later, König, Kuhlman, and Thienemann (1911) cultured a species of Phoma isolated from water, and although they secured pycnidia in a few

instances, they were unable to determine the conditions under which fruiting bodies developed, but they surmised that probably the lack of food supply was the causal relation.

Other related genera have been studied more or less, and detailed accounts of the growth and fruit-body formation of several species of Phomopsis on the ordinary laboratory media have been given. (Roberts, 1913; Harter and Field, 1913; Harter, 1914.)

Plenodomus destruens has recently been described by Harter (1913), who has cultured the organism upon the ordinary laboratory media, and has determined its optimum temperature. For the most part the above-mentioned articles, written from a phytopathological point of view, have used the pure culture as a device for furnishing material for pathogenic studies, and the description of the organism in culture is largely for diagnostic purposes.

METHODS OF INVESTIGATION

As *Plenodomus fuscomaculans* had shown no form of reproduction under the ordinary methods of culture (see p. 724), it seemed to afford an excellent opportunity to try the effect of various environmental factors as a test of the applicability of the methods of Klebs to phytopathological studies.

The strain of the organism used was the progeny of a single pycnidiospore, isolated by the dilution method. This strain had been tested and was known to be pathogenic to apple. In 1913 another isolation was made from a second collection of material, and a second strain obtained and similarly tested. In all later work both strains were used in all experiments. Aside from slight differences in vigor of growth, the cultures gave the same reactions.

All experiments were made in duplicate with each strain; hence, the experiments reported give results which are a summary from the record of at least two, and, in most cases, of four parallel cultures.

The glassware used, unless otherwise indicated, was the ordinary German glass. All glass culture dishes, when other than tap water was to be used, were cleaned by immersion overnight in cleaning fluid, followed by four rinsings of tap water and one rinsing of distilled water. When water of a higher purity than ordinary distilled water was to be used in the medium, the vessels were given an additional rinsing with the purer water.

The most commonly used culture dishes were small glass preparation dishes, or capsules, of about 35 c. c. capacity. These had a loosely fitting cover which rested upon a shoulder of the bottom.

The chemicals used were those of Kahlbaum. Solutions of various chemicals were made up as weight-normal solutions (1 molecular weight in grams in 1 liter of water); and where chemicals contained water of crystallization, this was added in computing the molecular weight.

The various nutrient media mentioned were made according to the ordinary formulæ. Prune-juice agar was made by using 75 gm. of prunes with 20 gm. of agar per liter. Pea, corn, and oat broth were made by autoclaving two seeds or grains of each in 10 c. c. of distilled water.

The tap water used in some experiments had a conductivity of approximately 400 to 600×10^{-6}, while the conductivity water averaged 2×10^{-6} at the time of preparation. This water was obtained either by distilling ordinary distilled water in a block-tin still or by double distilling such water in Jena glass. As is generally recognized, ordinary distilled water varies greatly in quality, but the conductivity of the distilled water used was probably within 4 to 12×10^{-6}.

The filter-paper used was Schleicher and Schull's, and, unless otherwise given, was No. 595. All media were autoclaved at approximately 15 pounds for 10 to 15 minutes, unless otherwise stated.

Inoculations, unless specified otherwise, were made with one drop of a spore suspension obtained by crushing pycnidia in a water blank. This was then filtered through a filter paper into a sterile test tube. The filter paper was sterilized in a test tube drawn out to make a funnel. This gave a device by which large masses of mycelium and pycnidia walls could be strained from the suspension. The spore suspension was added to the various cultures by means of a sterile bulb pipette equipped with a long, small-bore outlet.

EARLY EXPERIMENTS WITH ORDINARY LABORATORY METHODS

The organism brought into pure culture was grown upon ordinary laboratory media. This work was done in the spring and fall of 1911 at the Michigan Agricultural College, at a table at the rear of a large laboratory lighted from one side. Cultures were made in Petri dishes, flasks, and test tubes. Standard agar, prune-juice agar, apple stem and bark agar, apple twigs, parsnips, corn meal, potato, carrot, bean pods, beef broth, and filter paper, without other nutrients, as well as with various nutrient solutions, were the media employed. Cultures were grown under a variety of conditions, such as room conditions (test tubes in cans or in wire baskets), in the incubator at 25° C., and in the ice box at temperatures ranging from 7° to 13°. A few cultures were grown at 37.5°. On all the media mentioned growth was obtained, with more or less difference in color or vigor, but in no case were fruiting bodies of any sort produced. In some cases the cultures were allowed to dry out gradually; in other cases sterile water was added from time to time. Flasks of corn meal, with an abundant water supply, were set away in a cupboard for three months in an attempt to secure fruiting bodies in the time-honored way. In spite of this variety of trials, the organism remained a typical "sterile fungus," of which a number have been reported in literature.

But the organism, when inoculated into the host, gave characteristic lesions and typical pycnidia from which the organism could again be isolated. These reisolations were repeatedly tested, with results parallel to those obtained from the parent culture. Certain fungi—e. g., *Botryosphaeria ribis* and *Rhizoctonia* spp.—are known to fruit exclusively upon the host, and evidence seemed to point to this organism as one of that type.

EXPERIMENTS UNDER CONTROLLED CONDITIONS

In 1913, experiments were begun at the University of Michigan laboratory. In this work an attempt was made to find the effects of varying environmental factors, or, in other words, to analyze the formative as well as the inhibiting factors involved in growth and reproduction.

CONDITIONS FOR GROWTH AND REPRODUCTION

PHYSICAL FACTORS

LIGHT

The influence of light upon organisms has been recognized for a long time. Fries (1821) and the early authors attributed great morphogenic power to light. They found their greatest substantiation of the effect of light upon organisms in the excessive growth of mycelium in caves, accompanied, as it was, by the suppression of fructification. The literature is full of these observations, many of which are quoted by Elfving (1890). Scientific experiment with light as a factor influencing growth and reproduction of fungi began with the classic studies of Brefeld (1877, 1881, 1889) on *Coprinus* spp. Brefeld found in some species a complete suppression of fructification when cultures developed in the dark; in other species fructification took place, but the growth was puny. In some the high temperature of the summer replaced in part the beneficial effect of light. In a set of interesting experiments Brefeld showed that the exposure of mycelium to light need not be long (two to three hours) in order to have fructification begin, and that cultures so exposed developed normally, although in the dark. The work of Brefeld substantiated that of the older observers. Lakon (1907) has attempted to show that the action attributed to light is really due to transpiration differences in the cultures of *Coprinus* spp.

Downes and Blunt (1878) had previously experimented with the effect of light upon bacteria and found that it had a very detrimental effect upon these organisms. This they attributed to the action of the ultraviolet rays in augmenting oxidation, a property of light long recognized by chemists. Their conclusion was later substantiated by Ward (1893).

Elfving (1890) gave the results of his experiments with light in a monograph on the subject. Searching the literature, the only important experimental work found was that of Brefeld (1877, 1881, 1889) already

mentioned. Many had studied the effect of light upon germination, but the varying intensities of light used, etc., yielded nothing in the way of a generalization.

Elfving (1890) sought to find the influence of light upon metabolism. He used cultures of *Penicillium* spp. and a related fungus (*Briaraea* sp.) growing in a synthetic solution. He used several sources of carbon and nitrogen. Basing his conclusion upon the dry weights obtained in the light and in the dark, he decided that light acts upon fungi as an inhibitor of organic synthesis. The closer the food material is to protoplasm in its make-up, the less the light inhibits. This produces the result which he finds analogous to conditions in the higher plants—that light restricts vegetative growth. Elfving, in view of the great similarity of fungi in their physiological relations, boldly makes his conclusions apply to the whole group of fungi.

Lendner (1896) tested the effect of light upon species of Mucor, Botrytis, Amblyosporium, and Sterigmatocystis, finding that light was effective only under conditions of unfavorable nutrition.

Finally, in the experiments of Ternetz (1900) with *Ascophanus carneus*, asci were produced only under the influence of light.

Light is seen to be a factor of widely varying importance for organisms, although the effect on vegetative growth is commonly shown to be prejudicial. For some it is a morphogenic factor of great influence; for others it is of no moment.

Pure cultures of the organism on prune-juice agar and on parsnip had been brought from the Agricultural College laboratory. At Ann Arbor these cultures began to produce pycnidia in a few days. When analyzed, this striking behavior showed that light was probably the factor concerned with the fruit-body formation. The following experiments were started to test the validity of this inference. While work at the Agricultural College had been done some distance from the window (25 to 30 feet), the cultures at Ann Arbor were placed a few feet from a south window in strong diffuse daylight, and at times in direct sunlight.

Experience had shown that the organism would make a fair growth on filter paper. Filter-paper disks, about 5 cm. across, were folded to form cones, and these were set up in 10 c. c. of tap water in preparation dishes. These were autoclaved. To some, one drop (1/20 c. c.) of a sterile *M/1* chemical was added, as indicated in Table III. The preparation dishes were inoculated with a mycelium suspension, and were placed in tall battery jars covered with filter paper. One set of cultures was placed in a light-tight cupboard, while the other was left upon the table in strong diffuse light. Thermometer readings showed at times of strongest light that the illuminated cultures were 2 degrees centigrade warmer than those in the dark. Readings were made in nine days.

TABLE I.—*Effect of light: Tests with filter paper (readings in 9 days)*[1]

Conditions.	Number of pycnidia.	Growth.
Filter paper in light	+	++
Filter paper in dark	−	++[2]

[1] In tables where a single plus symbol (+) is contrasted with the negative sign (−), presence or absence is meant. Where a series of readings is given and several plus symbols are used with reference to pycnidia production, they give the average of two and at times of four readings, as follows: + = 1 to 10 pycnidia; ++ = 10 to 25; +++ = 25 to 50; ++++ = 100. As applied to growth the same plus symbols mean, respectively, scant, fair, good, abundant growth.

[2] A trifle stronger than above.

The cultures which had been in the dark were exposed to light about an hour at a time, when the reading was made. A second observation after 27 days showed the following result:

TABLE II.—*Effect of light: Tests with filter paper (readings in 27 days)*

Conditions.	Number of pycnidia.	Growth.
Filter paper in light	+	++
Filter paper in dark (except one hour's exposure)	Sclerotia.	+++

These bodies, called provisionally "sclerotia," when examined under the microscope were found to be minute brown bodies about one-tenth the size of the ordinary pycnidium and consisted of a firm, solid pseudo-parenchyma.

In no case was any suggestion of chamber formation noticed; nor were any spores found. It is noteworthy that the growth after this longer period could be seen to be stronger in the dark than in the light.

As part of the same experiment, a drop of some sterile *M*/1 chemical was added as indicated to a number of similar filter-paper cones. The results are as follows:

TABLE III.—*Effect of light: Pycnidium formation on filter paper plus various chemicals*

Chemical.	9 days.		27 days.		40 days.	
	Light.	Dark.	Light.	Dark.	Light.	Dark.
Filter paper + approximately 1/20 c. c. of—						
Calcium nitrate, $Ca(NO_3)_2$ *M*/1	+	−	+	Sclerotia.	+	Sclerotia.
Potassium acid phosphate, KH_2PO_4 *M*/1	+	−	+	−	+	+
Potassium nitrate, KNO_3 *M*/1	+	−	+	−	+	Sclerotia.
Calcium acid phosphate, $Ca(H_2PO_4)_2$ *M*/1	+	−	+	−	+	−

At the time of making the first reading, the cultures were exposed to the light for about an hour, and at the second reading they were exposed to strong diffused daylight for two hours.

From a consideration of the experiments reported in these tables, it is evident that light is a factor directly concerned with pycnidium production. There is also a strong tendency toward increased growth in the dark.

The experiment has been repeated many times, with a great number of duplicate cultures (60 in one instance), and always with similar results. The following is a typical experiment. Preparation dishes with water distilled out of sulphuric acid and filter paper and with water alone were inoculated with spores of each of the two strains of the organism. One set was wrapped in a double thickness of paper such as is used in photographic film rolls. The dishes exposed to light were set in glass battery jars on the window sill. The light was made diffuse by a sheet of yellow manila paper tacked on the window. The dark cultures were set away from the window in the interior of the room. The difference in temperature was the reverse of the conditions in the preceding experiments, since closeness to the cold window more than compensated for the effect of the light. In this experiment after a month no pycnidia formed in the dark, while in every culture in the light numerous pycnidia were found.

TABLE IV.—*Effect of light: Test with two strains of the organism*

Strain and conditions.	Pycnidia.		Growth.	
	Light.	Dark.	Light.	Dark.
Strain I:				
Filter paper + water	25	0	++	++++
Double-distilled water	2	0	+	+
Strain II:				
Filter paper + water	11	0	+	+
Double-distilled water	0	0	+	+

To avoid the criticism that the results observed were due to differences in aeration brought about by wrapping the capsules, or by the use of the dark closet, and to test other conditions of food supply, cultures were made with corn broth, and these were placed in a specially constructed light-tight box, which, however, allowed aeration. The box was made of two tubes of different diameters (7 and 9 inches), one inside the other. These cylinders were each 12 inches tall and toothed at the ends. A pair of caps were made for these cylinders. The caps consisted of a disk of paper about 10 inches in diameter, and a short cylinder 8 inches in diameter was glued to it. The joint was made light-tight with black paraffin. When these tall cylinders were set up with the cylinders of the caps fitting between them, light was excluded. The cultures were

placed in battery jars. The toothed tops of the cylinders allowed a circulation of air. For tests with light, the cultures were ordinarily placed in a battery jar and covered with filter paper or cloth to protect them from dust. As a further safeguard from error, however, a similar container was made, but with celluloid substituted for black paper.

The result with corn broth, after three weeks, is given in Table V.

TABLE V.—*Effect of light: Test with corn broth in light-tight box*

Conditions.	Pycnidia.	Growth.
Light in battery jar	+++	Fair.
Light in celluloid chamber	++	Fair.
Dark in black-paper chamber	−	Strong.

From this experimentation it is evident that light is a determining factor for pycnidium formation in this organism, irrespective of the type of nourishment, and that the action of light is distinct from effects which might be attributed to faulty aeration in the darkened cultures. The slight depression of pycnidia formation in the slightly darkened celluloid chamber is significant. Growth is increased in the dark.

Cultures on corn broth, in both light and dark, were subjected to a variety of air conditions. Stoppered flasks were fitted with two glass tubes, one of which extended to the surface of the culture, the other merely through the cork. As indicated in Table VI, some were connected with the water pump and filtered air which had bubbled through water was gently drawn through. As a check, some flasks were left with no additional circulation, while some were plugged with cotton.

TABLE VI.—*Effect of air circulation: Test with corn broth in stoppered flasks*

[Time, 1 month[1]]

Conditions.	Pycnidia.	Growth.
Attached to aspirator:		
Light	++++	++
Dark	−	++++
Air only through small tubes:		
Light	++	++
Dark	−	+++
Flasks plugged with cotton:		
Light	++++	++
Dark	−	+++

[1] The experiment was continued a second month with no change in relative values.

This experiment eliminates any possibility that the effect attributed to light may have come from faulty aeration or deficient transpiration. The experiment further has significance from the point of view of aeration.

The production of sclerotia, as recorded in Tables II and III, after a short exposure to light, and the production of pycnidia in one case, where the exposure was not more than two hours, suggested that the exposure to light did not need to be of long duration in order to produce its morphogenic effects. The capsules of a preceding experiment, which had shown no pycnidia after three weeks in the dark chamber, were divided into series, one of which was exposed to strong diffuse light on the window sill for two hours, while the other series was continued in the dark box. The exposed cultures were returned to the box, and after a week the cultures were examined.

TABLE VII.—*Effect of light: Continued test with corn broth*

Corn broth.	Mature pycnidia.	Growth.
Dark	0	Aerial growth.
Dark, light (2 hours), dark	3–4	Aerial growth checked, mycelium matted.

Pycnidium production had not increased upon a second examination a week later.

This experiment teaches that pycnidium formation is not only associated with light, but that the effect of light is to inaugurate a type of growth which can proceed to completion even in the absence of light. But after exposure to light the number of fruiting bodies formed is limited and the process does not continue to the production of a large number of fruiting bodies.

To summarize the results of this series of experiments, it may be pointed out that light is a decisive factor, which determines, in certain cultures, whether reproduction takes place or not, and that the action of this factor is irrespective of the richness or the poverty of the substratum in nutrients. As a morphogenic factor, its action is to inaugurate fruit-body formation, but it is not essential to the process, once inaugurated. Associated with its effect in initiating reproduction, we have its repressing effect on growth.

All subsequent cultures made with the organism had good exposure to strong diffuse light, unless otherwise expressly stated.

TEMPERATURE

It has been said that the influence of temperature was very early recognized in its influence on the life processes of fungi. Raulin (1869) in his studies of *Aspergillus niger* grew the organism at the most favorable temperature—33°. Wiesner (1873) very early formulated the behavior of *Penicillium glaucum* by a law which took into account that the time necessary for fructification did not depend wholly upon the

temperature at which a culture was placed, but depended also upon the temperature at which the organism had developed, which is, of course, a way of saying that the process of fruit-body formation is a process which depends upon the previous metabolism, and that conditions which delay the latter react similarly upon the former. The literature teems with individual facts about the temperature relation (Behrens, 1905, p. 444–449). The temperature relation, better than any other, shows the significance of the cardinal points in relation to life processes. Accordingly, we have the generalization of Klebs (1900), that the limits permitting vegetative growth are wider than those permitting fructification, and this law is nowhere more admirably illustrated than in the temperature relation.

My early experiments with temperature are not applicable, because light was excluded. Experience had shown that pycnidia were formed at the ordinary limits of room temperature. Successful cultures on various sorts of media were made in the winter with the average room temperature, 20 to 23°, and in the summer with a temperature range from 25 to 30°, so long as the light factor was not neglected.

A series of temperature experiments was made with the synthetic solution described upon page 752 in 100 c. c. flasks. These flasks were inoculated, and after three weeks' growth in weak diffuse light were subjected to the temperature indicated.

TABLE VIII.—*Effect of temperature*

Temperature.	How obtained.	Number of pycnidia.	Increase in growth.
°C.			
6–6½	Constant temperature ice box with glass doors	0	Slight.
10–12	Located at window in cold hallway	+	Fair.[1]
20–22	Room temperature near window	+	Strong.
23	Constant temperature incubator, outer door open, glass door closed	0	Weak.
33	do	0	Do.

[1] Pycnidia began to form after a week.

The varying conditions in this experiment make necessary some interpretation for the clearing away of the apparent contradictions in the results. The absence of pycnidia in the 23° and 33° incubators, which is in seeming contradiction to the production of pycnidia in the summer time, or even at ordinary room temperature, was doubtless due to the fact that either the light was too much reduced or the air was depleted of oxygen. That the former influence was not operative seems likely from the fact that cultures standing in battery jars upon the incubator had at another time produced pycnidia. The incubators contained other cultures at the time of the experiment, and, although the doors were opened from time to time, the chamber had the ordinary strong odor of old cultures. The constant low-temperature chamber

which was designed especially for this work seems free from this criticism, since cultures placed in it before icing began developed pycnidia. This incubator had two openings (1-inch diameter) to the outside and a small fan, driven by a motor, which continuously brought about good aeration and prevented fogging of the doors. The constancy of temperature during the first week can be vouched for within the limits set, and for the next month no large deviation occurred.

The lack of apparatus to give constant temperatures, and at the same time illumination and aeration, prevented any further experimentation along this line. Pycnidia have been obtained in cultures with a temperature range of from 10° to 30° C. No pycnidia were obtained at 6° C. and no other inhibiting factor than temperature is known to have entered. The experiments with the constant-temperature incubators are disregarded because of the entrance of other factors, but are included merely to show the difficulty of experimenting with this factor.

The wide limits of pycnidium production, so far as temperature is concerned, allowed great leeway in experimentation; but outside these limits temperature may show as marked an effect as light. It is noteworthy that growth shows wider temperature limits than reproduction.

AERATION

The oxygen relation is no doubt the most essential of all life relations, and the statement "No life without air" has been shown to be universal, the contributions of Beijerinck (1893), as well as those of Fermi and Bassu (1904, 1905), showing that even the strictest of known anaerobes require minute traces of free oxygen. The relation of oxygen to plants was recognized almost from the beginning, but the interpretation of respiration by Pfeffer (1889) is fundamental. In this we have respiration portrayed as the energy-releasing process. Subsequent work has dealt with the effect of various external conditions upon the respiratory quotient. Necessarily all respiration relations depend upon the quality of the nutrition as well as the quantity of nutrients. The general conclusion which has been expressed by Beijerinck (1899), that all plants have a definite oxygen optimum and that aerobes are those whose optimum is high, while anaerobes are organisms whose air requirement is low, seems to summarize most nearly the numerous contributions.

The limiting effect of scanty aeration upon reproduction has already been mentioned. Determination of the potency of this factor in any but general ways is difficult, because of other factors involved.

Observation very early showed that greater pycnidium production took place in a capsule or Petri dish than in a plugged test tube, and that small test tubes were not so effective for pycnidium production as larger ones. Similarly, when capsules were piled one on top of another in a battery jar, pycnidia production took place in the top capsules first, although in a few days or a week pycnidia were formed in all.

If a vigorous culture on suitable media (prune-juice agar or corn-meal agar) was sealed with sealing wax no pycnidia were produced, even though comparison tubes unsealed produced pycnidia in abundance. Sealed tubes which had remained without pycnidia for two weeks had the sealing wax removed, and the pycnidium formation was slowly inaugurated. Corn broth in capsules, if covered with a small bell or if placed in a battery jar with a tight-fitting ground-glass cover, produced scanty mycelial growth but no pycnidia.

Tests for aerotropism were made with spores in melted agar. Melted agar was heavily sown with spores of the organism. Some tubes were prepared with a lighter seeding. Small drops of these agars were placed on sterile slides and sterile cover glasses pressed down upon them. Other preparations were made with the cover glass tilted, as in Beijerinck's (1893) well-known experiments. These slides were put away in a moist chamber for 24 hours at ordinary room temperatures. The results of these tests were extremely interesting.

Where the spores were numerous those at the center of the preparation showed no evidences of germination other than a slight swelling. Outside the center zone germination became more and more evident. About 5 mm. from the edge of the cover glass the germ tubes were found to be 10 to 50 times the length of the spore. At the edge of the cover glass the germ tubes had extended outward nearly a half of a millimeter. Where the spores were fewer in number the germination in the center sometimes proceeded to the extension of a short germ tube. There was no evidence of a definite tropism toward the border of the cover glass, but frequently the same spore would have sent out two germ tubes from opposite sides, one growing toward the edge of the glass, the other growing inward. Then it was noticed that the sprout growing in the medium with the richer oxygen supply was from 4 to 10 times the length of the other germ tube.

Where a clump of spores occurred about halfway from the center to the edge of the cover glass, those spores near the edge swelled strongly and put out germ tubes, while spores of the same clump, situated nearer the center, remained dormant, or at least swelled only slightly. The repression of germination in these spores seemed to be related to the scanty oxygen supply, and for this there was strong competition.

A series of flasks of different sizes was prepared with filter-paper cones, wet with 5 c. c. of distilled water. These were autoclaved and inoculated with a spore suspension. Immediately after inoculation the cotton plug was pushed slightly down the neck of the flask and the flasks were sealed with melted paraffin. The flasks were set in a window in even, diffuse illumination. After a month the reading shown in Table IX was obtained.

TABLE IX.—*Effect of aeration: Test with flasks of different sizes*

Size of flask.	Number of pycnidia.	Growth.
C. c.		
50		None.
100		None.
250		Doubtful.
500	0	Weak.
1,000	0	Fair, mycelium blackish.

There were no checks in this experiment, but the behavior of this organism on filter paper had been so constant as to leave little doubt of repression of pycnidia having taken place, owing to the sealing of the flasks.

A similar experiment was performed with a number of nutrient solutions, some of which were known to allow pycnidium production, and others of which were known to yield only strong growth. Ten c. c. of each solution were used. This experiment was done in duplicate and was carefully checked. Inoculation was made with small masses of mycelium. The flasks, after inoculation, were sealed and stood in strong diffuse light upon a table. Table X gives the summary of this experiment.

TABLE X.—*Effect of aeration: Tests with various nutrient solutions*

[Time, 1 month]

Solution.	Size of flask.	Sealed.		Check.	
		Number of pycnidia.	Growth.	Number of pycnidia.	Growth.
	C. c.				
Raulin solution [1] (levulose substituted for sucrose).	900	0	Heavy mat.	0	Heavy mat.
	500	0	Heavy mat.	0	Heavy mat.
	125	0	Fair.	0	Heavy mat.
	50	0	Scant.	0	Heavy mat.
Acid Dox [2] solution with 1 c. c. of glycerin added to each flask.	900	0	Scant.	0	Fair, white.
	500	0	Scant.	0	Fair, white.
	125	0	Scant.	0	Fair, white.
	50	0	None.	0	Fair, white.
Alkaline Dox solution with 1 c. c. of glycerin added to each flask.	900	0	Very scant.	0	Weak.
	500	0	Very scant.	0	Weak.
	125	0	Very scant.	0	Weak.
	50	0	None.	0	Weak.
Raulin solution.	900	0	Fair.	0	Heavy mat.
	500	0	Fair.	0	Heavy mat.
	125	0	Fair.	0	Heavy mat.
	50	0	Fair.	0	Heavy mat.

[1] Raulin solution: 1,500 parts of water; 70 parts of cane sugar (35 gm. levulose); 4 parts of tartaric acid; 4 parts of ammonium nitrate; 0.6 part of ammonium phosphate; 0.4 part of magnesium carbonate; 0.6 part of potassium carbonate; 0.25 part of ammonium sulphate; 0.07 part of zinc sulphate; 0.07 part of iron sulphate; 0.07 part of potassium silicate.

[2] Dox solution, etc. (Czapek): Distilled water (H_2O), 3,000 c. c.; magnesium sulphate ($MgSO_4$), 1.5 gm.; dibasic potassium phosphate (K_2HPO_4), 3.0 gm.; sodium chlorid (KCl), 1.5 gm.; ferrous sulphate ($FeSO_4$), 0.03 gm.; with Potassium acid phosphate (KH_2PO_4), acid solution (Thom, 1910).

TABLE X.—*Effect of aeration: Tests with various nutrient solutions*—Continued

Solution.	Size of flask.	Sealed.		Check.	
		Number of pycnidia.	Growth.	Number of pycnidia.	Growth.
	C. c.				
Raulin solution + ½ c. c. *M*/1 calcium nitrate ($Ca(NO_3)_2$).	900	0	Fair, mat.	10	Fair, thin mat.
	500	0	Fair, no mat.	10	Fair, thin mat.
	125	0	Fair.	25+	Mat.
	50	0	Fair.	25+	Mat.
Raulin solution with levulose, on filter paper cones.	900	0	Filter covered.	0	Paper covered.
	500	0	Filter covered.	0	Paper covered.
	125	0	Less than above.	0	Paper covered.
	50	0	As above.	0	Paper covered.
Acid Dox solution + 1 c. c. *M*/10 arabinose.	900	5	Scanty white.	25+	Fair, white.
	500	1–2	Scanty white.	25+	Fair, white.
	125	0	Scanty white.	50+	Fair, mat.
	50	0	Scanty white.	50+	Fair, mat.
Acid Dox solution + 1 gm. potato starch.	900	?	Strong.	20+	Strong, mat.
	500	?	Strong.	20+	Strong, mat.
	125	0	Fair.	20+	Strong, mat.
	50	0	Fair.	20+	Strong, mat.

This experiment shows the effect of scanty aeration in repression of growth as well as an almost complete suppression of pycnidia in the sealed flasks. In the two cases where pycnidium production did take place in the sealed flasks, the fructification occurred in the larger flasks of the series. It must be said that the check flasks, especially the larger sized ones, were almost dry at the close of the experiment and the humidity conditions as well as the concentration were different from those of the sealed flasks. For the first three weeks, however, the cultures were approximately the same, and it seems safe to attribute the difference in growth and pycnidium suppression to improper aeration, rather than to the drying or concentration, especially since, as will be seen from later experiments, these factors play but little part in pycnidium production.

From the many observations recorded here, and from the experiments, it seems safe to conclude that this organism is very sensitive to the oxygen supply, and it requires good aeration for optimum growth and for pycnidium production.

HUMIDITY (TRANSPIRATION)

From a number of indications in cultures, it was felt that transpiration might be a factor of more or less importance in the growth and reproduction of this fungus. A study of the literature dealing with reproduction, especially the work of Klebs (1898) with *Sporodinia grandis*, made this

seem extremely probable. It was seen that cultures on various complex media did not produce pycnidia until they began to dry out, as a general rule. Moreover, on nutrient solutions the pycnidia commonly form on the surface. On vegetables, such as carrot or parsnip, or on prune-juice agar, the pycnidia formed in the aerial mycelium.

Very early this relation was suspected as being operative, and the filter paper cone was used in the first experiments to further transpiration and aeration. When, however, the relation was tested, it was seen that the actual formative influence of transpiration had been greatly overestimated. Filter-paper cones were compared with similar-sized disks of filter paper entirely submerged. Inoculation was made with bits of mycelium, and the cultures stood on the table in strong diffuse light.

TABLE XI.—*Effect of humidity: Test with filter paper*

[Time, 1 month]

Conditions.	Number of pycnidia.	Growth.
Cones mostly above water	5–10	Fair.
Submerged paper	10+	Scanty.

It is seen that the pycnidia production goes on after this period as strongly, if not better, in the submerged condition, while the growth seems slightly stronger on the cone. Since differences of this sort are hard to estimate, little importance is attached to the slight differences. Nevertheless, we have in this experiment striking evidence that under conditions where transpiration is reduced to the zero point pycnidium production is nevertheless vigorous.

In this experiment the possible relation to contact stimuli is not avoided. The following observation is even more conclusive, for here contact relations are limited to the effect of mutual contact of the threads of the mycelium itself, and no further elimination of a hypothetical contact relation is possible. Several water blanks of ordinary distilled water were heavily inoculated with spores and mycelium, respectively. After a month the following observation was recorded:

TABLE XII.—*Effect of humidity: Test with inoculated water*

Form of inoculation.	Number of pycnidia.	Growth.
Spores	4–10	Fair amount of white, byssoid mycelium. Total submergence.
Mycelium	2–10	Fair amount of white, cottony mycelium. Total submergence.

From these experiments there can be little doubt that pycnidia can be produced by this fungus without reference to the factor of transpiration.

We now come to an experiment in which the time element was recorded and in which the influence of a number of different degrees of air humidity was tested.

Four bell jars with a hole in the top were connected with a compressed-air reservoir so that a gentle current of air could be sent through the apparatus. The air was led into the bell jars by a tube reaching to the bottom of the bell jar and taken out by short tubes which extended through the stopper but a short distance. To secure moist conditions the air was bubbled through distilled water, while dry air was obtained by sending the blast through two towers filled with calcium chlorid. The first bell jar received moist air constantly, the fourth dry air constantly, and the second and third were connected by Y tubes to both the dry and wet bell jars, so that they could be made to receive either wet or dry air independently. Throughout the experiment the conditions in these two bell jars were alternated. The second bell jar received wet air for three days and then dry air for one day, while the conditions were reversed for the third jar. Preliminary tests with a Lamprecht polymeter in each jar (these were set to agree with a sling psychrometer reading) showed that the humidity within the first jar ranged from 65 per cent to 70 per cent, and in the fourth the humidity was only 20 per cent. In the other bell jars a humidity of 65 per cent or a dryness corresponding to 25 per cent could be obtained in a half hour by blowing in wet or dry air. The blast was almost continuous throughout the experiment except for a period each day between about 3 a. m. and 8 a. m., at which time the pressure was lacking. The bell jars giving wet conditions were fogged at times, but, as the apparatus was in strong light and as the fog disappeared except when the bell jars were hit by a cold draft, it is very likely that the light intensities were sufficient in all cases. For media various substances were used. Bits of pear and apple twigs, corn meal, slices of carrot and apple, peas, rice, and corn, as well as corn-meal agar and glucose agar, were autoclaved. The media were prepared in capsules without the covers and were placed in tiers in round wire baskets so that each capsule had free access to air. The basket was slipped inside a battery jar and was covered with a cotton pad held in place by a glass plate. Five sets of this sort were prepared, four to be subsequently placed under the bell jars, and the fifth to be used as a check without aeration. The media were autoclaved and then inoculated with a drop of spore suspension to each dish. The cultures were left one week under ordinary room conditions. At the beginning of the test of the various air conditions the bell jars were drenched with solution of mercury bichlorid. The basket was lifted under aseptic precautions and set upon a small metal rack. This

rack, which had been previously disinfected, rested upon the ground-glass base. The bell jar was quickly put in place over the basket and sealed air-tight by the use of anhydrous lanolin. Since the air pressure at times amounted to several pounds, these bell jars had to be clamped to the base plate. This was accomplished by boards drilled at the corners, the top one fitted with a 3-inch hole, through which the top of the bell jar projected. Long bolts fitted with thumb screws held the boards in place and thus when tightened prevented the jars from leaking. The air was filtered through cotton before it reached the cultures. Several times during the experiment the cultures subjected to dry air were moistened with a few cubic centimeters of water. It was found that those cultures were nearly dried out at these times.

No pycnidia were formed with peas, rice, or glucose agar under any of the conditions. Other cultures showed the pycnidia in the same relative proportions for the various conditions of aeration. The record for corn broth may be cited as typical.

TABLE XIII.—*Effect of humidity: Test with corn broth under bell jars*

[Time, 30 days]

Medium.	Number of pycnidia.					Growth.				
	Unaerated.	Wet.	Mostly wet.	Mostly dry.	Dry.	Unaerated.	Wet.	Mostly wet.	Mostly dry.	Dry.
Corn broth.....	0	0	0	+	+++	+++	++++	++++	+++	++

Pycnidia had been formed for some time before the reading was made. The aeration was continued, and a month later another reading was made. At this time all the cultures except peas, rice, and glucose agar showed pycnidia, irrespective of the air condition, with the exception of the series left as a check. This series, left in a battery jar, covered with a cotton pad and a glass plate of the same size as the jar, made good growth, but in no case did pycnidia occur.

We have in this experiment results which indicate that at most the effect of moist air is to delay pycnidium formation. Whether this effect is due to decrease in transpiration or to nutrition conditions, either of the substratum or of the aerial mycelium, brought about by the excess of water in the air or condensed upon the hyphæ is not known, but it seems likely that the water relation is the most potent one, since with such efficient aeration the transpiration must be considerable in all cases. The previous experiments indicated that absence of transpiration was not directly inhibiting to pycnidium formation with cultures which were under conditions of scanty nutrition. The last experiment reiterates that conclusion, but indicates that the humidity may serve to delay fruit-body formation. The effect of moist air in delaying but

not suppressing pycnidium formation is always associated with increased aerial growth. When it is recalled that with rich media the pycnidia are commonly formed in the aerial mycelium, this opposed condition may be significant. Further discussion of this behavior is given at another place (page 741).

In conclusion, it may be pointed out that transpiration, or, better, low air humidity, is a factor of only secondary or contributing influence in fruit-body formation for this fungus, and in no sense is a positive determining factor like light or aeration.

Physicochemical Factors

Reaction of the Substratum

The acid or alkaline reaction of nearly all biological fluids—the blood, milk, sea water, cell sap—varies but slightly from neutral. It is commonly said that fungi grow best under slightly alkaline conditions. Many organisms show great tolerance to either alkalinity or acidity, but the organism here investigated showed a comparatively narrow range, and its optimum point was not that of the great group of fungi, but much more like the optimum for bacteria.

The following experiment with filter-paper cones and with Raulin solution shows something of the limits of growth and reproduction for this organism. The acidity or alkalinity [1] indicated in the table was obtained by the addition of either normal potassium hydroxid or hydrochloric acid (potassium hydroxid in case of the Raulin solution, since it was acid at the outset).

Table XIV.—*Effect of acidity and alkalinity: Test with Raulin solution and filter paper*

[Time, 1 month]

Reaction.	Raulin solution.		Filter paper.	
	Number of pycnidia.	Growth.	Number of pycnidia.	Growth.
−10		Contaminated		None.
− 5		None		None.
0	+	Strong	20	Scant.
+ 5	++	Strong	20	Scant.
+15	0	Fair		None.
+28	0	Fair		None.

This experiment showed the strict relation of this organism to the chemical reaction, both as to growth and as to reproduction, and, as usual, the growth limits were wider than the limits of reproduction. The experiment also revealed why Raulin's solution had previously

[1] Computed in terms of cubic centimeters of normal hydrochloric acid or potassium hydroxid in a liter by titrating 5 c. c. with *N*/20 standards, phenolphthalein as indicator.

given growth but no fruiting bodies. Once this relation of the organism to acid and alkali was known, previous experiments could be reviewed in the light of it and the behavior of certain chemicals explained.

Ten c. c. of a 5 per cent gum-arabic solution was autoclaved in a series of preparation dishes. The solution received sterile chemicals to give concentration as shown in the table and was inoculated with a spore suspension.

TABLE XV.—*Effect of acidity and alkalinity: Test with various chemicals*

[Time, 1 month]

Chemicals.	Concentration.	Reaction.	Number of pycnidia.	Growth.
Gum-arabic solution plus—				
Potassium acid phosphate	*M*/200	+	++	++
Potassium acid phosphate + Sodium acid phosphate	*M*/200 each	+	+++	+++
Sodium acid phosphate	*M*/200	+	++	+++
Dibasic potassium phosphate	*M*/200	−	o	+++
Check		±	+	++
Sodium hydroxid		−5	o	+

This experiment, if it be permitted to draw conclusions by comparison of salts with a similar anion or cation, indicated that the specific effects in pycnidium formation were not due to any specific ion, for if potassium were the influential ion, then we should get no effects with the similar sodium salt. More conclusive still was the effect of the dipotassium phosphate as contrasted with the dihydrogen salt. Here the same ions were concerned, but in different proportions. The experiment shows the extreme sensitiveness of this organism to alkalinity, since a reaction of −5 was sufficient to cause absence of pycnidia.

A study of the reaction of some of the common media, as given in Table XXV, shows how reaction controls not only reproduction, but growth as well. Of the complex media tried the most favorable for pycnidium production was a couple of corn grains autoclaved in 10 c. c. of water. Aside from the nutrition relation, which will be discussed later, the acid reaction is largely responsible for the excellence of this medium; but the time when this reaction is most effective is at the period when growth has covered the medium, not the mere reaction at the start. Corn broth shows at the start an acidity of +8, and after a month the reaction is still acid, +5. As is seen from Table XIV, this is a favorable condition for pycnidium production. Pea solution at the beginning of a period of culture showed an acid reaction of +8, while oats showed at the start a reaction of +5. The latter showed after a month a reaction approximately neutral. It will be seen from Table XXV that oats were a correspondingly poorer medium than corn. Pea broth, on the other hand, showed a reversal of condition, and after a month

titrated −8. The culture grew vigorously for a week or two, formed a mat and some aerial mycelium, then the gradual checking of the growth occurred. The culture ceased producing aerial mycelium and the mat became half submerged. Soon all growth ceased and the culture grew but indifferently or not at all when transplanted.

If old pea-broth cultures were acidified to approximately +5 with potassium acid phosphate, tartaric acid, or hydrochloric acid, growth started again and pycnidium production took place upon the dense mat.

Other media showed similar changes in either acid or alkaline reaction, and, as a rule, it may be said that media with a proportion of protein lower than the carbohydrate proportion show after a period of growth an acid reaction (Wehmer, 1891). With media high in protein the reaction becomes alkaline (Nägeli, 1880).

The consideration of the acidity or alkalinity of substrata at the start and at the close of a period of culture leads naturally to a consideration of autointoxication. This is especially appropriate in this case, since the autointoxication effects observed were due to harmful reactions produced by the by-products of metabolism. These by-products were not of the complex type commonly thought of in connection with the term autointoxication, but were mostly the simple and well-known end products of carbon and nitrogen dissimilation. The injurious effects were produced to a large extent by the acidity or alkalinity engendered, and the same effects could be artificially produced in a favorable medium by mere change of reaction.

Depending upon the excess of carbohydrate or protein, as has been said, the reaction of the substratum became either acid or alkaline. In the case of excess of carbohydrate, oxalic acid is formed by this organism, and in old cultures of corn, oats, or prune-juice agar crystals of calcium oxalate were often found. In the case of protein excess, as was demonstrated for old pea-broth cultures, the medium contained an excess of ammonia. This ammonia could be detected by boiling the liquid from such old cultures and testing the fumes with a strip of wet, red litmus paper.

In a solution where the carbohydrates and protein constituents are present in a proper ratio, these by-products of metabolism neutralize each other. Corn broth is a notable example of this type of medium, for in it the by-products, even after two months, are not potent enough to interfere with reproduction.

The action of these autointoxication products in the substratum is further illustrated by the common experience met with in transferring from old cultures of this organism. In old agar cultures of various sorts the mycelium was found dead when it was submerged in the substratum, although the aerial mycelium remained alive for more than a year.

We have, therefore, in autointoxication a phase of the major factor, acid or alkaline reaction, and while definite harmful bodies of a protein or amid type are known for organisms and may have been present here, we have in the end products of protein and carbohydrate dissimilation harmful constituents whose influence may be to limit either growth or reproduction.

Chemical Factors

Quantity of food

The quantity, rather than the quality, of the food needed for this organism can more conveniently be considered at the outset. As was stated at the beginning of the experimental work, there is a certain minimum for growth and also for reproduction. Naturally, reactions taking place at the base level of nutrition are sharper and less obscured than those taking place where food is in abundance and the factors of reaction, autointoxication, etc., have greater and greater influence. For this reason, once the capacity of this organism to grow and reproduce upon material almost devoid of nutrients was recognized, many of the experiments with other factors have been performed with the food supply reduced to a low level.

This power to grow upon simple stuffs and with them in extremely high dilution naturally led to the question of the minimum essential. Growth and reproduction in distilled water has already been mentioned. The distilled water used in the first experiments was the ordinary distilled water of the laboratory. The glassware used was "resistance," cleaned as described. The test tubes were plugged with cotton, and a few motes of cotton could be seen upon the surface of the water after inoculation. Inoculation was made as described with a spore suspension. The number of colonies which resulted from inoculation with similar-sized drops of this suspension in Raulin solution was from 5 to 20. These details show that a very small amount of organic stuff was introduced from the inoculum. After three or four weeks a white or gray filmlike mycelium could be seen, either attached to the glass or floating near the bottom of the test tube. After a month or, at times, two months 2 to 5 pycnidia were produced under the water.

It is difficult to understand where the carbon and nitrogen used by the fungus came from. The minerals might be accounted for more or less satisfactorily by assuming that they came from the glass, which is slightly soluble. For the organic stuffs we have a few possibilities. The nitrogen may have come from ammonia in the air, and the carbon from the small bits of cotton dropped from the cotton plug. It is more than likely that the distilled water carried some oily volatile material, which, while not strongly influencing conductivity, gave a suitable foodstuff for the fungus. Or we have the possibility, first pointed out by Elfving (1890), that organisms may be fed by small quantities of volatile sub-

stances which are absorbed from laboratory air by the water (Beijerinck and Van Delden, 1903). Be the source of this food supply what it may, I was interested to find if all distilled water, even the purest, had enough food supply or absorbed enough to support both growth and reproduction.

Conductivity water[1] of a value 3.03 times 10^{-6} was used as in the preceding experiment, with, however, the following improvements in the method. Jena glass test tubes were used throughout. The test tubes were plugged with long-fiber absorbent cotton, and the preliminary dry sterilization, which has a tendency to make the fibers brittle, was omitted. Inoculation was made with one drop of a filtered spore suspension which had about 25 to 50 spores to the drop. The pycnidium which furnished these spores was growing in aerial mycelium, so none of the old substratum was brought over. At all events, material brought with the spores was diluted nearly 200 times. After two months slight growth was evident as faint submerged wisps or skeins. The growth was less than a tenth as strong as that produced in ordinary distilled water. No pycnidia were formed.

This experiment indicates that in the soluble glass and in the character of the distilled water we have the important sources of the food supply. The motes of cotton were practically eliminated in the last experiment. It might be thought that the nutrition in this case was as good as the preceding—assuming the food supply to come from volatile chemicals—and that the poor growth of mycelium and the failure to reproduce was due to the toxicity of the conductivity water. But the toxicity of ordinary distilled water is generally admitted to be greater than the toxicity of conductivity water. Moreover, this organism has never shown any effects which might be attributed to toxic substances in the water. In the recent experiments on the toxicity of distilled water with other plants the nutrition phase has been neglected, since the conclusions have been drawn from tests with the well-nourished roots of seedlings. In the experiments here reported, the food supply carried in the plants is that which is within a few spores barely visible with the high power of the microscope. It is difficult therefore to attribute the effects to anything but the scantiness of nutrition.

The conclusion, therefore, is drawn that while growth and reproduction can take place with the meager food supply of ordinary distilled water in "resistance" glass, the limit of reproduction is reached with conductivity water and Jena glass, but the limit of growth is still lower.

This same relation to nutrition was shown with the following experiment with filter paper. It had been determined in many previous experiments that this organism could grow and reproduce upon filter paper and distilled water. Tests with tap water, distilled water, and conductivity water indicated that the material used for growth and

[1] I am indebted to Dr. R. P. Hibbard, of the Michigan Agricultural College, for the conductivity water. The measurements of resistance were also made by him.

reproduction came largely from filter paper. Although filter paper is said to be the purest form of cellulose obtainable, Schwalbe (1910–11, p. 600) states that appreciable amounts of oxycellulose and hydrate-cellulose are present. Since filter paper is known to have some ash, a preliminary experiment was performed to find if this ash served, in part at least, as a source of food. A pair of culture dishes was prepared with a filter-paper cone in each. Ten c. c. of ordinary distilled water were added. To each of two other dishes with a similar amount of water, the ash from a filter cone was added. These dishes were autoclaved. Inoculations were made with spores. After three weeks the results shown in Table XVI were obtained.

TABLE XVI.—*Effect of quantity of food: Test with filter paper and the ash from filter paper*

[Time, 3 weeks]

Medium.	Pycnidia.	Growth.
10 c. c. distilled water, plus filter cone	+	Good.
10 c. c. distilled water, plus ash	−	Scanty.

The better growth and the pycnidial production on the filter paper, as opposed to the results with ash, indicate that the influential stuffs are not those from the ash. It may be remarked that the readings were taken early enough to avoid complications due to the slow pycnidium formation in distilled water. The effect of ash having been shown to be negligible, the main experiment was set up. Five sheets of filter paper (S. & S. 595) about 15 cm. across were autoclaved in 500 c. c. of conductivity water in a Jena flask. This furnished a stock solution, which was diluted with conductivity water by means of pipettes and graduates, which were carefully rinsed before and during the operations. The dilutions were prepared in Jena beakers, but were eventually put in 10 c. c. quantities in a number of Jena test tubes. These were autoclaved and inoculated with a spore suspension. This experiment was done in duplicate with each of the strains of the fungus, with the results shown in Table XVII.

TABLE XVII.—*Effect of quantity of food: Test with filter-paper broth*

[Time, 2 months]

Medium.	Pycnidia.	Growth.
Filter-paper broth:		
1/1	+ (3)	Fair, easily seen.
1/100	+ (1)	Fair, easily seen.
1/1,000	+ (1)	Scant, barely visible.
1/10,000	−	Scant, barely visible.
Conductivity-water check	−	Scant, barely visible.

The experiment shows that the ordinary high-grade filter paper, when autoclaved with water of high purity, yields sufficient nutriment for growth and reproduction of this organism. A dilution of 1/100 is still sufficient for pycnidium production, but at 1/1,000 we have reached the limit of food supply sufficient for pycnidium production. Growth, as usual, takes place at greater limits than reproduction.

This experiment gives conclusive evidence that the toxic substances of distilled water do not affect this organism. We may now conclude that we have been working nearer and nearer the limits of growth and reproduction. The amount of material required is evidently extremely minute. It is in the imponderable mass of stuff, somewhere between distilled water and conductivity water, or in that bulk of stuff lying between 1/1,000 and 1/10,000 dilution of a filter-paper broth.

Having now some conception of the extremely low limits of concentration at which growth may take place, we may now consider the growth and reproduction relations at higher concentrations.

The experiments already reported give a mass of details as to growth, at various concentrations, but no conclusions from these isolated cases are justified, because the reaction is so masked by other relations.

The following experiments allow a comparison of some nutrient solutions at various concentrations. The solutions chosen were those which did not become toxic with the continued growth of the organism. In one experiment 200 grains of corn were autoclaved in 1 liter of tap water. This solution was concentrated to approximately 100 c. c. by boiling in a beaker. It was, therefore, approximately 10 times the strength of ordinary corn broth. The strong solution was also diluted as shown in the table. Cultures were made as usual and were inoculated with a spore suspension. The results are shown in Table XVIII.

TABLE XVIII.—*Effect of quantity of food: Test with corn solution*

[Time, 1 month]

Concentration.	Pycnidia.	Growth.	Remarks.
10X	−	+++++	White.
5X	−	++++	White.
1X	+++	++	Blackened.
1/10X	+	+	Blackened.

In this experiment it is seen that the organism, after a month, produced fruiting bodies only in the lower concentrations, but the growth was strong in the higher concentrations. The growth in the weaker concentrations had increased but slightly after the first two weeks. We may conclude then that a food supply which allows a fair growth and then becomes exhausted is most favorable for pycnidium formation.

The following experiment with synthetic media was performed. The combination described upon page 752 was made up at 25 times the usual

concentration. This was diluted as shown in Table XIX, and cultures were made as in the preceding experiment.

TABLE XIX.—*Effect of quantity of food: Test with synthetic solution*

[After 1 month]

Dilution.	Pycnidia.	Growth.	Remarks.
25X.....	—	o	
10X.....	—	o	
5X.....	o	++++	White or pinkish.
2X.....	o	+++	Black mat formed.
1X.....	Many.	+++	Slightly less growth than above, black mat.
1/2X.....	Many immature.	++	Slightly less growth than in 2X. Abundant evidence of pycnidia starting.
1/5X.....	10	+	Growth weak.
1/10X	5	+	Pycnidia extremely minute. Mycelium scanty.

The experience with this solution shows that doubling the concentration of a favorable culture solution increased growth, and was sufficient to inhibit completely pycnidium formation. A solution diluted one-half gave promise of many pycnidia—more than in the 1X concentration—but the pycnidia were slow in forming. In the extremely low concentration growth was scant and a small amount of pycnidium production took place. The experiment leads to the same conclusions as the preceding experiment—i. e., that a limited food supply is essential to fruit-body formation, and the optimum concentration is one which gives a comparatively large mycelial growth before the exhaustion takes place.

The teaching of this experiment would place the limit of concentration of a sugar at *M/100*. We have, however, a great body of experiments already outlined in which pycnidium production took place with a sugar concentration considerably higher. For instance, in Table X pycnidia are reported for Raulin's solution (cane sugar *M/7*) when a calcium salt was added. Or, considering the experiments with corn grains, these seem to present a contradiction when it is noted that the pycnidia were first formed on the corn grain with its rich food supply. Similarly, the various laboratory media—such as prune-juice agar, parsnips, and carrots—all are rich in carbohydrates; yet these are reported as allowing pycnidium production.

In these rich solutions, however, an extremely abundant aerial mycelium is produced, and as the medium begins to dry the pycnidia are produced in the aerial strands, but never upon the medium itself. In a few cases a dense mat formed over the agar, and this effectively walled off the new food supply. On only one laboratory medium—corn-meal agar (Shear and Wood, 1913)—were the pycnidia produced directly upon the agar. It is noteworthy that with this medium the mycelium production is scant. In the case of corn grains the pycnidium production does not take place until the corn grain is dried somewhat, and this, coupled with

the fact that the corn grain is not extremely soluble, accounts very well for the appearance here. Instead of the corn grain furnishing nutrition, the corn grain soon becomes the location where food supply is soonest exhausted. In this behavior upon drying, we may also find the explanation of the behavior of the wet and dry bell jars reported in Table XIII. The behavior of the 1× and ½× concentrations of the synthetic medium may be considered in this connection. It seems that in this case we have a similar factor to deal with. The mycelium in these concentrations grows at the top of the solutions, a trifle submerged in the case of the weaker solution. The stronger mycelial growth in the higher concentration leads to the formation of a thicker surface film in it than in this weaker one, and the film starts much sooner. The pycnidia are produced upon this surface film, which, no doubt, in some ways interferes with the utilization of the food supply.

From this it would seem that the limiting concentration suggested—*M/100* for sugar—instead of being too low is doubtless too high, and the production of pycnidia at this concentration, at the period stated, is brought about by the other factors, which lead to an even greater reduction of the available concentration.

When we consider the action of this aerial life of the mycelium in fostering reproduction, we find that our knowledge of the transfer of materials in mycelium is extremely limited. It, however, seems very likely that with the increase in concentration in the medium below and the drying of the threads, the diffusion of foodstuffs to the aerial parts is interfered with.

QUALITY OF FOOD

MINERALS.—The work with the quantity of foodstuffs just outlined indicates the extreme difficulty of determining what minerals are essential for growth. This sensitiveness to extremely small amounts, which doubtless is paralleled by other organisms, makes experimentation with ordinary methods or ordinary chemicals unreliable. The problem of determining the necessary mineral elements for this fungus would be impossible with our present technic.

An attempt was made to find the effect of certain chemicals when they were added to various nutrient solutions. Although many experiments were performed, the results were so masked or influenced by the constituents of the medium that no conclusions could be drawn. Notable influences which have been explained as other than nutrition effects have been obtained with acid phosphates and with calcium compounds.

The behavior of one chemical, magnesium sulphate ($MgSO_4$), is worthy of record. Since Molisch's accurate work (1894), this substance has generally been regarded as essential in fungous cultures. The following experiment suggests that the chemical may have a profound effect upon fructification. Two preparation dishes each received 10 c. c. of a solution

containing magnesium sulphate in $M/33$ concentration. Conductivity water was used. Inoculation was made with a drop of spore suspension. After one month many (more than 50) pycnidia were found in the loose submerged mycelium.

As a mineral base for nutrient solutions, monobasic potassium phosphate and magnesium sulphate, along with other chemicals, were frequently employed. The net result of numerous cultures made in the attempt to find some hint of the value of this or that mineral was the conclusion that cultures with these two constituents alone, with a suitable nitrogen and carbon supply, gave as good results as more complex combinations.

This solution of mineral salts contains the bulk of the elements generally considered essential for fungus growth. Carbon and nitrogen need to be added to secure the complete nutrient, but iron can be neglected, since it is such an unavoidable impurity in chemicals and is usually present as a constituent of the glassware. Beijerinck (Samkow, 1903) had used a similar solution as a culture medium for bacteria.[1]

Because of the extremely small amounts of minerals found necessary for growth and reproduction in this form, I modified the formula by cutting down the concentration of the various components. Since the solutions were to be used in comparative work, the chemicals were added on a molecular-weight basis. At the time of the first experimentation it was thought that the reaction should be approximately neutral, and accordingly molecularly equivalent weights of potassium acid phosphate and sodium carbonate were employed. Similarly, through dependence upon relations of other plants, it was thought that magnesium sulphate might be slightly toxic, and it was used at a lower concentration than either of the other two minerals. The solution thus devised for preliminary experiments contained sodium carbonate and potassium acid phosphate as $M/100$ and magnesium sulphate as $M/500$. Subsequent experiment showed that the carbonate could well be omitted and the magnesium sulphate increased from fivefold to tenfold.

The other combinations were used for comparison with this mineral base. The mineral constituents of Raulin solution and those of Dox solution were tried, and while either were suitable, neither had any advantage over this modified Beijerinck solution; on the contrary, they were much more complex and contained the mineral elements in excess of the needs of this fungus.

CARBON SUPPLY.—The carbohydrates form the common source of carbon for fungi. Other classes of compounds, as pointed out by Nägeli (1880) and Wehmer (1891), may be utilized. For this organism, as indicated in Table XXIII, other classes of compounds—but of alcoholic

[1] Samkow used the following base with a great variety of organic compounds: Potassium acid phosphate, 2 gm.; sodium carbonate, 2.5 gm.; magnesium sulphate, 0.4 gm.; water, 1 liter.

structure—may be utilized as a carbon source (malic acid and glycerol). As is well known, various plants possess widely varying amounts of sugars, and the sugars and other carbohydrates differ markedly in kind. The specific effects of certain vegetable media have been attributed by many to the specific action of the type of carbohydrate furnished. Roux and Linossier (1890), as a result of their work with the fungus *Dematium albicans* Laurent, announced as a general biological law that with an increase in the molecular weight of the carbohydrates the complexity of the growth form of the fungus increased. With certain sugars, such as glucose in a 1 per cent solution, these investigators obtained only yeastlike growth, but with a biose, such as maltose, they obtained strong mycelium and conidia production. Recently Hiekel (1906), repeating the work of Roux and Linossier, but with 10 per cent sugar solutions, accepted the conclusions of the French investigators within certain limits. A priori, it is very difficult to see why two sugars, such as glucose and maltose, should differ in specific effects, since the latter, when hydrolized, yields only the former.

Very early in the investigation tests were made with the common sugars to find whether there was a specific effect on fruit-body formation due to the various sugars. In these tests the sugars used were used as weight-normal solutions; hence, the effects secured were not obscured by concentration differences. The various sugars were added from a sterile stock *M/1* solution to 10 c. c. of the autoclaved nutrient solutions, as indicated in the table. Glass preparation dishes were used, and all were placed in strong diffuse light. Inoculations were made with spore suspension in the usual manner. The tests were done in duplicate. Table XX shows the average of conditions.

TABLE XX.—*Effect of quality of food: Test with sugars*

Sugar.	Pea broth.			Oat broth.			Tap water and filter.		
	Sugar concentration.	Pycnidia.	Growth.	Sugar concentration.	Pycnidia.	Growth	Sugar concentration.	Pycnidia.	Growth
Saccharose	M/10	0	+++	M/10	0	+++	.....	.....	.....
Do	M/20	0	++++	M/20	0	+++	.....	.....	.....
Do	M/50	0	+++	M/50	0	+++	M/50	0	++
Dextrose	M/10	0	.....	M/10	0	+++	.....	.....	.....
Do	M/20	0	++++	M/20	0	+++	.....	.....	.....
Do	M/50	0	+++	M/50	0	+++	M/50	0	++
Levulose	M/10	0	+++	.....	.....	.....	.....	.....	.....
Do	M/20	0	++++	.....	.....	.....	.....	.....	.....
Do	M/50	0	+++	.....	.....	.....	M/50	+ (1 or 2)	+
Maltose	M/10	0	++++	.....	.....	.....	.....	.....	.....
Do	M/20	0	++++	.....	.....	.....	.....	.....	.....
Do	M/50	0	+++	.....	.....	.....	M/50	0	++
Check	.....	0	++	.....	+	++	.....	+	+

It will be noticed that in nearly every case, even in low concentration of sugar, there was an increased growth following the addition of sugar. Filter paper and oat broth, which normally produce pycnidia, gave strong growth with saccharose, dextrose, and maltose, but no pycnidia. In the case of levulose *M*/50, the growth was not greatly increased, and one or two pycnidia appeared. This number is much less than the normal for filter paper alone. We may conclude that these sugars exert a repressing influence on pycnidium production, and at the same time augment vegetative growth. How this is brought about is difficult to explain; but in some way the ratio of the constituents was so altered that the limits for reproduction of some factor—e. g., reaction—or of some group of factors was exceeded.

A more comprehensive experiment was performed in which a large number of carbohydrates was tested. Equal parts of the minerals of Raulin's solution in 2 × concentration were added to various *M*/10 sugar solutions and to 2 per cent solutions of the polyoses whose molecular weight is not known. Each combination was set up in four capsules, using 10 c. c. per dish. The media were steamed on three successive days and inoculated with a drop of spore suspension for each dish. Table XXI gives the result of this experiment.

TABLE XXI.—*Effect of quality of food: Test with carbohydrates*

[Time, 2 months]

Carbohydrate.	Concentration.	Size of colonies.	Growth.	
			Character.	Form of fructification.
		Mm.		
Xylose (pentose)	*M*/20	3–4	Compact	Oidia.
Maltose (disaccharose)	*M*/20	3	Compact	Oidia.
Glucose (monosaccharose)	*M*/20	2	Compact	Oidia.
Mannose (monosaccharose)	*M*/20	2	Compact	Oidia.
Galactose (monosaccharose)	*M*/20	2	Compact	Oidia.
Levulose (monosaccharose)	*M*/20	1–2	Compact	Oidia.
Arabinose (pentose)	*M*/20	1–2	Compact	Oidia.
Sorbose (monosaccharose)	*M*/20	(2¼)	Floccose	Pycnidia.
Sucrose (disaccharose)	*M*/20	½–1	Compact	Oidia.
Raffinose (polysaccharose)	*M*/20	2–3	Floccose, very loose	Mycelium.
Lichenin (polysaccharose)	1 per cent		Loose mat, covering dish.	Secondary spores.
Dextrin (polysaccharose)	1 per cent		Diffuse mat, covering dish.	Pycnidia.
Inulin (polysaccharose)	1 per cent		do	Pycnidia.
Gum arabic (polysaccharose)	1 per cent		do	Pycnidia.
Gum tragacanth (polysaccharose).	1 per cent		do	Pycnidia.
Wheat starch (polysaccharose).	1 per cent		No growth	
Lactose (disaccharose)	*M*/20		No growth	
Erythrose (tetrose)	*M*/20		No growth	

In the above table the sugars and other carbohydrates are arranged on the basis of vigor of vegetative growth. In the main the results of the former experiment are substantiated. The strongest growth took place with the highly soluble sugars, and the dishes were filled with small ball-like masses. The strongest growth was not associated with pycnidia production, but on the contrary was opposed to it. At first glance the law of Roux and Linossier (1890) seems operative, for pycnidia appeared in the carbohydrates, which are known to have extremely high molecular weights. But this superficial agreement is abundantly contradicted by the first part of the list. Without regard to molecular weight, these sugars gave approximately the same growth form, and the variation in amount of growth was not striking. It will be noted that these sugars are highly soluble, while those toward the bottom of the list are almost insoluble. In the one case every bit of the foodstuff was available, while in the other only a slight amount of the carbohydrate was open to appropriation. The preceding experiment with filter paper and sugars proved that, where the scant available carbohydrate of filter paper allowed pycnidia production, the addition of sugars destroyed the balance between growth and reproduction, and only growth took place. The same general relations exist between the members in this table as existed in the former experiment. It is worthy of note that Roux and Linossier (1890) and later Hiekel (1906) drew their conclusions from carbohydrates such as the first seven. We can find in their method of work the source of their error. Their solutions were made up on a percentage basis, and where they drew a conclusion that a complex sugar like maltose in 1 per cent solution gave a more complex growth than a 1 per cent glucose solution, because of the difference in molecular weight, they were in reality comparing *M/36* and *M/18* solutions, and their conclusion really applies to concentration. They had previously shown that a low concentration would call out more complex growth forms.

The cause of the variation in growth among the various sugars is not known. A great many factors undoubtedly enter. Nearly all the sugars used were split in approximately the same way by the various specific enzyms of the organism. Differences in absorption rates, in rapidity of enzymotic action, etc., may enter and be responsible for the differences in growth here recorded. It may further be remarked that although the sugars used were of the highest purity they vary in their relative freedom from contamination, owing to difficulties in separation and purification. The colloidal carbohydrates undoubtedly carry a mass of adsorbed material, while in the others, traces of calcium, nitrogenous material, etc., may be present. It is not unusual to find a minute gummy scum on freshly prepared maltose solution.

Certain other interesting points are to be found in the table. The production of the growth form called "oidia"—multiseptate, heavy-walled hyphæ resembling Dematium or at times Monilia—were constantly

found in the highly soluble sugars. Such growth forms have commonly been recognized as a reaction to high osmotic pressure. Ternetz (1900) has obtained these in acid solutions. But such growth forms have occurred with this fungus in distilled water and on filter paper, and no doubt this growth form, instead of being a specific reaction to concentration, is one induced by a number of unfavorable conditions.

The action of sorbose has been disregarded, because this sugar is broken down by heat. The failure to obtain growth with lactose and erythrose is not without parallel in the literature. The action of wheat starch is peculiar, in view of the previous successful use of potato starch (Table X).

The action of lichenin is of great interest. This carbohydrate is a dextrin-like compound, almost insoluble in cold water and forming a gummy mass in hot water. In the turbid solution of this chemical the fungus produced a great number of secondary spores, evidently hyphomycetous. These spores were of approximately the same size and shape as the ordinary spores of this fungus. The exact method of their production was not determined. Mounts of material gave only straight mycelial threads and great numbers of detached spores. Dilution plates poured from the culture dishes teeming with these spores gave no other organism than the one under investigation. The colonies appeared in the plates in such abundance as to leave no doubt concerning the relation of these colonies to the secondary spores.

The experiments with carbohydrates may now be summarized. Nearly all carbohydrates tried served as a source for carbon. The general effect of adding sugars even in so low a concentration as $M/50$ was to stimulate vegetative growth greatly, but this stimulated growth was accompanied by a pronounced repression of pycnidium formation. In an experiment with $M/20$ solutions a strong mycelial growth was obtained, accompanied by oidia-like bodies, but fructification was absent. With slightly soluble carbohydrates, in which the actual amount of available soluble material was always limited, vegetative growth was weaker and pycnidium production was a general rule. A comparison of these highly soluble and slightly soluble carbohydrates indicates that the differences in growth form are connected with the amount of food supply rather than with the specific nature of the sugar. This position is reinforced when we consider that the hydrolysis of inulin, gum arabic, etc., yields exactly those sugars which, when tested in $M/20$ concentration, gave no pycnidia. In view of this comparison the earlier conclusion of Roux and Linossier (1890) seems untenable, and a more plausible explanation of the differences of growth form obtained seems to be found in the concentration relations.

This matter of carbohydrate supply has obviously a marked influence upon the problem of the organic media for laboratory use.

NITROGEN SUPPLY.—That the organism was influenced by the kind and amount of the nitrogen supplied seemed evident from the results of experiments with standard media, such as beef broth and beef agar, as well as the results already reported for pea broth.

A number of preliminary experiments of the same type as those reported under carbohydrates were performed at the same time, and these indicated that the various nitrates influenced pycnidium formation. But these results were not altogether consistent. The following experiment (see Table XXII) with filter paper and tap water plus various chemicals, and the similar series in which distilled water was used, may be cited as typical.

TABLE XXII.—*Effect of quality of food: Test with various nitrates*

[Time, 1 month]

Chemical.	Present as—	Distilled water.		Tap water.	
		Pycnidia.	Growth.	Pycnidia.	Growth.
Calcium nitrate	*M*/100	20–30	++	20–50	+++
Potassium nitrate	*M*/100	50–100	+++	50–100	+++
Calcium acid phosphate ($Ca(H_2PO_4)_2$) + calcium nitrate.	*M*/200, *M*/100	30–50	+++	100+	++
Potassium acid phosphate + potassium nitrate.	*M*/100 each	.....	+++	100+	+
Filter paper	Check	9–20	+	7–12	+

From this experiment it could not be determined beyond question that the nitrate ion was the potent factor in this increase in pycnidia formation, but the corresponding behavior of both the calcium and the potassium nitrate indicated that this was extremely likely. The increase in pycnidium production upon the addition of both a phosphate and a nitrate to this carbohydrate medium is significant.

Since the nature of the carbon assimilation might greatly influence the nitrogen assimilation, experiments with these two compounds can hardly be separated. In the following experiment an attempt was made to test various classes of carbon-furnishing compounds with various nitrogen sources. In this experiment the mineral solution mentioned in the preceding section was used. The stock solution contained monobasic potassium phosphate as *M*/100, sodium carbonate as *M*/100, and magnesium sulphate as *M*/500. To different portions of this, malic acid, glycerol, and maltose were added, respectively, so that each chemical was present at *M*/100 concentration. A fourth series was prepared as a check, and in this cones of filter paper furnished the carbon supply (S. & S. 605). The various solutions were put into series of preparation dishes, 5 c. c. per dish. To these dishes the nitrogen compounds to be tested were added from a clean pipette 1 drop (1/20 c. c.) of the proper solution (stock solutions were made up *M*/50, except peptone, which was 2 per cent) to

each dish. The various combinations employed, and the dilutions present in the culture, are indicated in Table XXIII. In every instance the concentration given shows the amount of the chemical that was present in the culture. The experiment was done in quadruplicate.

TABLE XXIII.—*Effect of quality of food: Test with nitrogen and carbon compounds*

Stock solution of minerals plus—		Number of pycnidia.	Growth.
Carbon.	Nitrogen.		
Malic acid, *M*/100	Peptone, 0.02 per cent	100	Scant.
Glycerol, *M*/100		60	Strong.
Maltose, *M*/100		0	Strong.
Filter paper			No growth.
Malic acid, *M*/100	Asparagin, *M*/100		None.
Glycerol, *M*/100		5	Scant.
Maltose, *M*/100		100+	Strong.
Filter paper			None.
Malic acid, *M*/100	Leucin, *M*/600	5	Scant.
Glycerol, *M*/100		0	Scant.
Maltose, *M*/100		25–50	Strong.
Filter paper			None.
Malic acid, *M*/100	Potassium nitrate, *M*/500		None.
Glycerol, *M*/100		2–7	None.
Maltose, *M*/100		50	Fair.
Filter paper		0	Scant.
Malic acid, *M*/100		10–20	Fair.
Glycerol, *M*/100		0	Scant.
Maltose, *M*/100		0	Fair.
Filter paper		10–15	Fair.
Peptone		1–5	Scant.
Asparagin, *M*/500		0	Scant.
Leucin, *M*/600		0	Scant.
Potassium nitrate, *M*/500		0	Scant.

This experiment shows that nitrogen, as previously shown for carbon, may be taken from widely different classes of compounds. The availability of any particular nitrogen compound is largely determined by the associated carbon compound. For instance, peptone, which carries available carbon, gave a large number of pycnidia with malic acid, but none with maltose. Asparagin, which gives the best growth and the greatest number of pycnidia with maltose, gave no pycnidia with malic acid. Glycerol, which seems on the whole to be a poor carbon source, gave with peptone strong pycnidium production, but with other nitrogen compounds behaved indifferently. As a further complication, peptone is able to serve both as nitrogen and as carbon source. Leucin gave poor growth with all carbon compounds except maltose, and a comparison of its behavior with that of asparagin, which is a compound of the same class, is interesting.

The experiment shows in a striking way how unlimited the possible combinations of nutrients may be. The marvelous thing is the absolute regularity of the product, regardless of this or that varied food supply. Growth that morphologically could not be distinguished arose from a protein or a mineral nitrate. Pycnidia were produced from these widely divergent compounds, with carbon compounds equally separated, and in these were billions of spores which did not differ in a manner permitting measurement, each a potentiality which could repeat indefinitely under these conditions the same reaction.

From this experiment we may pick a combination which is favorable for growth, but which also gives an abundance of pycnidia. For further experiments the combination of minerals with maltose and asparagin was chosen. The steps, more or less logical, which lead to the development of this synthetic culture solution may be reviewed. Experiment had shown that the essential mineral elements necessary for the growth and development of this fungus were contained in two mineral salts. Experiments in which these are added to various nutrient solutions give a hint as to the value and the concentration suitable. Eventually a compound was selected which gave the mineral salts which needed to be supplied and in addition had a chemical which could be used to make the reaction less acid, as desired. Previous work had shown that the organism could grow and produce pycnidia on extremely limited amounts of minerals, so the amounts taken were extremely small—far smaller than the ordinary formulas call for. In choosing the carbohydrate, wide choice was possible, since so many allowed good growth. Maltose was selected for use in the experiment just reported, because, next to xylose, it had given the best growth. The use of xylose was not advisable because of its high cost, but care was taken to use maltose in small amount, so that the effect found in the experiment reported in Table XX would not be repeated. Accordingly, *M*/100 concentration was provisionally chosen. The device for deciding upon the nitrogen source has been detailed in the preceding experiment. The low concentration of nitrogen was chosen to avoid such toxic conditions as were found in the pea broth. In passing, it may be said that an attempt was made to secure approximately the ratio of carbon to nitrogen that exists in the corn broth, which had been found extremely favorable to the organism.

Different concentrations of the separate constituents of this nutrient solution were further tested, with extremely interesting results. The device used was to vary the concentration of one constituent while holding the others constant. It was thought that in this way approximately the optima for all the constituents could be found.

The following experiment was performed with double-distilled water (slightly poorer than conductivity grade) and "non-sol" glass flasks. Dilutions were prepared as outlined in the table, and the culture media

steamed on three successive days. It was found that steaming instead of sterilization under pressure was very important. In a previous attempt the media were sterilized in the autoclave, and upon inoculation absolutely no growth took place. The experiment was done in quadruplicate with one strain. Inoculation was made with a spore suspension as before. The flasks were set in strong diffuse light near a window. Readings were made after a month. Five c. c. of water were added to each flask, and a second set of readings [1] were made after another month. The result of the experiment is shown in Table XXIV.

TABLE XXIV.—*Effect of quality of food: Test with synthetic solution in various combinations*

[Time, 2 months]

Medium.			Number of pycnidia.	Growth.
Potassium acid phosphate, *M*/100. Sodium carbonate, *M*/100. Maltose, *M*/100. Magnesium sulphate, *M*/500	Plus asparagin	*M*/50	0	±
		M/100	0	±
		M/500	50	++
		M/1,000	0	+
		M/5,000	0	+
Potassium acid phosphate, *M*/100. Sodium carbonate, *M*/100. Magnesium sulphate, *M*/500 Asparagin, *M*/500.	Plus maltose	*M*/10	0	+++
		M/50	0	+++
		M/100	50	++
		M/200	0	+
		M/1,000	0	+
Potassium acid phosphate, *M*/100. Sodium carbonate, *M*/100. Maltose, *M*/100. Asparagin, *M*/500.	Plus magnesium sulphate.	*M*/50	[a] 13	++
		M/100	1	+++
		M/500	50	++
		M/1,000	0	++
		M/5,000	0	+
Potassium acid phosphate, *M*/100. Magnesium sulphate, *M*/500 Maltose, *M*/100. Asparagin, *M*/500.	Plus sodium carbonate.	*M*/10	0	0
		M/50	0	+
		M/100	50	++
		M/200	90	++
		M/1,000	[b] 200	+++
Magnesium sulphate, *M*/500 Sodium carbonate, *M*/100. Maltose, *M*/100. Asparagin, *M*/500.	Plus potassium acid phosphate.	*M*/10	8	+
		M/50	0	++
		M/100	50	++
		M/200	0	±
		M/1,000	0	0

[a] 50 in 1.

[b] 295 in 1.

The device adopted is seen to be a very helpful one in determining the value of the various concentrations employed. The cultures in which asparagin was varied show how fortunate a concentration was chosen in the preliminary experiments. Similarly the experience with maltose shows that if asparagin is taken as *M*/500 then the maltose must have

[1] I am indebted to my colleague, Mr. J. H. Muncie, for making these readings.

approximately five times the strength. The experiments with magnesium sulphate are contradictory in part, but when the experience on page 742 is considered it may be concluded that for this organism the magnesium-sulphate ratio may be increased with profit. The phosphate proportion represented by *M/100* seems to be the favorable one. Sodium carbonate is found to be a constituent entirely unnecessary and for the most part detrimental to fruit-body formation.[1]

By this experiment, which could profitably be carried still farther within the limits indicated, a synthetic culture medium was obtained which gave for this organism a far greater pycnidia production than any other medium tried.

The merits of this medium may now be considered. It is a solution which contains the minerals necessary for growth of a vigorous character, but these chemicals are not present in superfluous amounts. It contains the carbohydrate which gave a remarkably strong, vigorous growth with this fungus, but the amount of the sugar is limited. The nitrogen source is a chemical of known composition and with maltose gave the strongest pycnidium production in the previous experiments. From the behavior of this organism we may conclude that we are approaching an ideal culture medium for the growth and reproduction of this organism. But we may go even farther, since the physiological relations of fungi to the substratum are so much alike. We can safely say that this combination will be found widely useful in producing similar reproduction in related forms. By the application of the same type of manipulation, some such combination can be found for other forms which will give better results than are now obtained on the ordinary media.

We may now consider some of the ordinary laboratory media in their effects upon this organism. The fungus has been cultivated upon a great many of the ordinary materials used in the laboratory for stock cultures and for diagnostic work. In this culture work the relation to light and to oxygen has been carefully observed. The relation to reaction has been but tardily recognized. The experience reported for pea broth shows that almost all relations to media can be reversed by changes in reaction (acidity or alkalinity). The initial relation is not, however, of as much importance as the reaction to phenophthalein after sufficient growth has taken place to lead to pycnidium formation. Table XXV summarizes the behavior of the organism on the complex media, with the relations on the synthetic solutions included for comparison.

[1] For convenience the amounts used in preparing this solution may be given. Stock solutions of *M/5* chemicals are prepared as follows:

Magnesium sulphate+7 Aq. 2.466 gm.+50 c. c. water.

Potassium acid phosphate 1.36 gm.+50 c. c. water.

Asparagin 1.33 gm.+50 c. c. water.

Maltose 3.60 gm.+50 c. c. water.

For 100 c. c. synthetic solution take 1 c. c. of *M/5* magnesium sulphate and 5 c. c. of each of the other solutions, and add to 84 c. c. water. Steam on three successive days.

TABLE XXV.—*Effect of quality of food: Complex media compared with synthetic solution*

Medium.	Reaction. Start.	Reaction. One month.	Growth character.	Aerial form.	Pycnidia.	Time.
Prune-juice agar (broth from 75 gr.).	+8	±	Strong white mycelium, becoming greenish. Medium reddened.	Strong tufted..	+++[a]	3 weeks.
Glucose agar[b] (glucose 3 per cent; peptone 1 per cent).	.....	.....	White, restricted growth becoming red-brown; oidia.	Prominent....	o	
Corn-meal agar (Shear)...	+10	+8	Weak growth of mycelium, mostly submerged; no mat.	Scant, if any..	++	4 weeks.
Standard agar.....	+15	−5	Strong growth, white, forming mat.	Scant, if any..	o	
Standard gelatin.....	+20	−5	Strong growth, white, gelatin slowly liquefied.	Fair amount..	o	
Filter paper.....	±	±	Scant amount creeping from point of inoculation, becoming greenish black, paper not discolored.	.....	++	1–3 weeks.
Parsnip plug.....	.....	.....	Strong, quickly covering plug, which shrivels. Color white, then tawny	Tufts.....	+++	4 weeks.
Carrot plug.....	.....	.....	As above, color greenish at close.	Tufts.....	+++	4 weeks.
Pea broth (2 seeds in 10 c. c.).	+8	−8	Strong, forming tough mat, which becomes submerged; white.	Scant.....	o	
Corn broth (2 grains in 10 c. c.).	+8	+5	Scant to medium amount, submerged, forming a film, otherwise no aerial growth, blackening at time of fruiting.	.....	++++	15 – 20 days.
Beans (2 seeds in 10 c. c.).	.....	.....	As in peas.....	.....	o	
Bananas (autoclaved)....	.....	.....	Strong, covering slice, reddish brown when old.	Strong.....	+	Slowly formed.
Rice plus 5× water.....	.....	.....	Strong, covering grains, which blacken after a month; white mycelium becoming gray-green.	Strong tufted .	o	
Oats (2 grains in 10 c. c.).	+5	±	Weak, submerged growth forming film; blackens in a month.	.....	+	4 weeks.
Raulin solution.....	+30	+20	Strong white, becoming tawny.	Strong tufted .	o	
Synthetic solution: Potassium acid phosphate, *M*/100. Magnesium sulphate, *M*/500. Maltose, *M*/100. Asparagin, *M*/500....	+30	+5	Good white growth, submerged, forming film on surface, on which pycnidia form; mycelium blackens just before fruiting.	.....	+++++	4–6 weeks.

[a] Aerial. [b] One formula calls for 200 gm. of glucose per liter (Harter, 1913).

A comparison of the media with reference to their reaction (+ or −) has already been made. In this relation we have a sharp determining factor which eliminates many preparations. Other media, such as rice, may be taken as cases where a poor balance exists between the nitrogen supply and the carbon supply, thus setting up an unfavorable toxic condition. The corn broth and the synthetic solution behave alike. The aerial growth seems to be strongest in substrata of an acid character. With rich substrata pycnidium production is aerial. The rapid production of pycnidia on filter paper is very significant. The wide range of suitable media is of great importance, and, since these substances must present carbohydrates and nitrogen compounds in great variety,

we have in these complex forms the same sort of result as was obtained in Table XXIII. But, in spite of the variety, the growth is much the same, and when fruiting bodies are produced they are the same morphologically. Such uniformity can be explained only by the assumption of an assimilation process which deals with much the same stuffs in all the substrata. The reserve materials are then worked over by the protoplasm under favorable conditions, and the fructification takes place.

Effect of Change of Intensity of a Factor during the Growing Period

Those experiments of Klebs (1899) in which a bit of rapidly growing mycelium of *Saprolegnia mixta* was transferred from a good nutrient solution to another of poorer quality, with resulting strong response in sporangium production, are the most striking demonstrations of the relation of checked growth to reproductive processes. In experiments of this type we have a device for studying some of the factors with the aim of their further simplification. We must, however, recognize that, no matter how ingeniously the term "checked growth" fits the phenomena described, it really tells us little about the physiological processes underlying.

The following experiment was performed. Strongly growing mycelium (1 week old on corn broth) was washed in two changes of 500 c. c. each of conductivity water. This mycelium was cut in pieces approximately the same size with sterile scissors and was added to the various sterile solutions shown in the table, with the results shown in Table XXVI.

Table XXVI.—*Effect of change of intensity of a factor: Withdrawal of food supply*

[Time, 1 week]

Medium.	Number of pycnidia.	Growth increment.
1-week-old mycelium added to—		
Filter paper	25	++
Conductivity water	5	+
Corn broth, 1/40	25	++
Corn broth 1×	0	++++
Magnesium sulphate, approximately *M*/100	2	+
Pea broth	0	+++++
Pea broth, 1/40	0	++
Check (similar mycelium allowed to grow undisturbed)	0	++

It is evident from these results that with the withdrawal of the food supply from vigorous, susceptible mycelium reproduction sets in promptly. The results were obtained in one week—two weeks after inoculation—although normally pycnidium production with corn grains is much slower. This hastening of the reproductive process by change of quantity of food supply indicates that here we were able to produce the change which takes place more slowly in the ordinary cultures.

The following experiment (see Table XXVII), which was performed as part of the experiment given on page 741, gives the effect of change of concentration upon the mycelium. The experiment was made with corn broth and with synthetic solution. The transfer was made after three weeks' growth had taken place.

TABLE XXVII.—*Effect of change of intensity of a factor: Change of concentration of food supply*

Extent of change.	Number of pycnidia.	Growth increment.
CORN BROTH		
From 10X to 10X	2	++
From 10X to 5X	25	+
From 10X to 1X	25	+
From 10X to 1/10X	25–40	+
From 5X to 10X	(a)	
From 5X to 5X		
From 5X to 1X	2–25	++
From 5X to 1/10X		
From 1X to 10X	50	++
From 1X to 5X	4–9	++
From 1X to 1X	0	+++
From 1X to 1/10X	12–15	+
From 1/10X to 10X		
From 1/10X to 5X	0–3	++
From 1/10X to 1X	0	++
From 1/10X to 1/10X		
SYNTHETIC SOLUTION		
From 10X to 1X	0	+++
From 10X to 1/5X	100	++
From 10X to 1/10X	20	+
From 5X to 25X		
From 5X to 10X	0	++++
From 5X to 2X	0	++++
From 5X to 1X	0	++ b
From 5X to 1/2X	100	+++
From 5X to 1/5X	50	++
From 5X to 1/10X	50	++
From 1X to 10X	25	+
From 1X to 5X		
From 1X to 2X	0	+++++
From 1X to 1/5X	0	++++
From 1X to 1/10X	0	++++
From 1/5X to 5X	15	+
From 1/5X to 1X	12	+
From 1/5X to 1/10X		
From 1/10X to 2X	0	++
From 1/10X to 1X	100	+++
From 1/10X to 1/2X	0	+
From 1/10X to 1/5X	0	+++
	100	++
	50	++++
	50	+

[a] These transfers were not made.

[b] Many immature.

The results given in Table XXVII show in striking manner the effect of the transfer of mycelium from one concentration to another. When mycelium from a poor solution is placed in a rich solution, it begins to grow vigorously, and, on the other hand, when rapidly growing mycelium is transferred to a solution of less concentration, the increase in growth is less. Exactly as the mycelium is checked or started into growth, reproduction is fostered or inhibited. While from the results of the experiments reported before it could only be said that these conditions of growth and reproduction occurred constantly side by side and therefore were related. From this last experiment we have definite proof of the interrelation of these two processes.

Other factors than food supply were experimented upon in the same way. The experiment previously reported under temperature (p. 726) strictly speaking belongs here. It may be remarked that pycnidium production began in the cold before it began in the cultures under room conditions. A similar experiment was performed with corn broth. Corn grains with mycelium about two weeks old, which showed no signs of pycnidium production, were set near a window at room temperature, and in the light in a cold attic where the temperature was about 10° C. After one week there were many pycnidia in the culture in the cold and the growth was checked, while in the culture under room conditions pycnidia production was just beginning and growth had continued regularly. After two weeks, however, the pycnidia were abundant in all the cultures, but were more abundant in the cultures under room conditions. From this experiment it is seen that a checking of growth by other means than food withdrawal can operate in much the same favorable way upon reproduction.

If, then, the factor light, which is known to have a strong power of checking growth, operates in influencing pycnidia production in this manner, we should be able to replace the light effect by checking the mycelial growth in some other way. Cultures, if left in the dark, ought to produce pycnidia eventually. Cultures with scanty food supply, such as those on filter paper, ought to yield pycnidia rather quickly in the dark. The experiments already reported have failed to show this action. Therefore, the action of light is not merely due to the checking influence which it has upon mycelial growth. If it were, we should have the paradoxical condition in which the withdrawal of light from a culture with limited food supply would augment pycnidium production, because of the greater growth in the dark and the more rapid diminution of the nourishment.

The following experiment (see Table XXVIII) was performed, in which the effect of checking the growth of corn-broth cultures by low temperatures was tried in both light and dark conditions. Corn-broth cultures 12 days old were placed under the conditions shown in the

table. The cultures in the dark were placed in the dark chambers described on page 723, and those in the light were placed in battery jars with tilted covers.

TABLE XXVIII.—*Effect of change of intensity of a factor: Change in temperature*

Conditions.	Pycnidia.		Growth increment, two weeks.
	One week.	Two weeks.	
Room temperature (22°):			
Dark	0	0	++++
Light	0–4	25–50	++
Approximately 10°:			
Dark	0	0	++
Light	10–15	10–25	++

The conditions were continued for two weeks longer without any change in the relations. This experiment reinforces the conclusion just arrived at that light has some other action than a mere checking of growth, and its action can not be replaced by a mere checking of growth.

Light is known to have a powerful oxidizing effect, and organic material under the influence of light is subjected, according to Freer and Novy (1903), to the action of organic peroxids engendered by the catalytic action.

The following experiment was tried to determine whether some such action was concerned. Hydrogen peroxid was added to 12-day-old corn-broth cultures at the rate of 2 drops (1/20 c. c.) of a 3 per cent solution to a dish. The experiment was checked with cultures of the same age. The dishes were placed in a dark chamber. After a week (first examination) the result shown in Table XXIX was obtained.

TABLE XXIX.—*Effect of change of intensity of a factor: Addition of hydrogen peroxid to corn broth*

Medium.	Pycnidia.
Corn broth + hydrogen peroxid (H_2O_2)	+[a]
Corn broth, check	−

[a] 4 to 8.

By strongly oxidizing cultures with hydrogen peroxid it was possible to replace the morphogenic action of light. Light, therefore, must act in some such manner upon this organism, and the action in fruit-body formation must be of some such character. This experiment was repeated at least six times, with varying concentrations of hydrogen peroxid. With cultures grown in the dark for from two to three weeks, the addition

of from 1/25 to 1/5 c. c. of hydrogen peroxid (3 per cent) would produce a few pycnidia with darkened cultures. In the stronger concentrations the mycelium was completely enveloped with a froth. After the first stimulation the cultures produced no further pycnidia. It must be said that in no case were pycnidia produced in amounts equal to those under light conditions. At best the use of hydrogen peroxid is a very harsh method.

With young cultures or with very old cultures the hydrogen peroxid was ineffective. In these its behavior is like that of light.

Other chemicals known to be strong oxidizing agents were employed. It may be said that nearly all gave positive results at extremely weak dilutions, provided that the mycelium used was in proper condition. Mycelium which would produce pycnidia by an hour's exposure to light gave good results with the oxidizing agents.

Another factor was doubtless responsible for the inequality of pycnidium formation in these experiments. All the chemicals used are toxic to the mycelium. In the concentrations used, these poisoned the cultures and certainly affected the reactions.

Table XXX summarizes the successful trials.

TABLE XXX.—*Effect of change of intensity of a factor: Use of various chemicals*

Chemical and concentration.	Corn broth.	Synthetic.	Pea.
Nitric acid (HNO_3), *M*/500	+	+	+
Sulphuric acid (H_2SO_4), *M*/500	+	+	−
Sulphuric acid (H_2SO_4), *M*/500, + potassium dichromate ($K_2Cr_2O_7$), *M*/500	+	+	−
Potassium permanganate ($K_2Mn_2O_7$), *M*/500	−	−	−
Ferric chlorid ($FeCl_3$), 1 drop of *M*/5	+	+	−
Zinc sulphate ($ZnSO_4$), *M*/500	−	−	−

GENERAL DISCUSSION

The work reported in this paper has given more or less of a definition of the environment in which *Plenodomus fuscomaculans* can live and reproduce. We now know the bare essentials for growth—the base level of existence—since we know the minima of the various formal conditions of growth. Similarly, we know some of the highest intensities which can be tolerated.

For growth at the base level of existence, there is only required the almost immeasurably small food supply of conductivity water, a scanty amount of free oxygen, and a temperature of 6° C.—perhaps lower. These factors may be increased in intensity until there is tolerated a food supply enormously larger, abundant oxygen, and temperatures up to 37° C.—perhaps higher. But as the simple minimum conditions are passed, the interactions of the component factors of the environment increase, and new factors arise which also have their limits. With

increase of food supply we must now consider, besides the mere chemical parts, the ratio of these parts to each other, both at the outset of growth and throughout the growing period. We analyze such relations and classify them as reaction, etc.

For pycnidium production the limits are found to be much narrower than those suitable for growth. No reproduction takes place at the base level of existence. Food supply must be increased, not greatly, but in measurable amount. From the scanty supply in conductivity water it must increase to the quantity found in distilled water—a two-fold to tenfold increase. Or it must be present in at least one thousandth of the quantity cooked from a few sheets of finest filter paper by conductivity water, but one-tenth of this amount is not sufficient. Oxygen must be present in abundance; stagnant air prevents reproduction. The temperature may be as low as 10° C., but must not be as low as 6½° C.

Up to a certain limit (perhaps up to *M*/50), increase in concentration of the food supply augments reproduction. After that point the excess food supply retards and eventually inhibits reproduction. Fructification, when it does take place with media of higher concentration, takes place in the aerial mycelium, and doubtless here the conditions are comparable to those in which the fructification is produced within or upon the medium.

The kind of food may vary almost without limit. An organism which can grow and reproduce in distilled water or a grain of corn can find requisite food materials in almost any biological product. But the more complex substances bring new relations, which, while of some importance to growth, are of decisive importance for reproduction. Growth can take place between the acid and alkali limits of +30 and −10 to phenolphthalein, but reproduction is limited to the conditions but slightly more acid than the neutral point of this indicator.

Corn broth seems at first glance a better foodstuff for this organism than oat broth, and in two parallel cultures the first will produce 50 pycnidia while the other is producing one. Yet if the oat culture be acidified with an acid phosphate, or even with hydrochloric acid, it becomes nearly as good a culture medium as the corn. Glucose agar made after the ordinary formula gives a strong growth with this organism, but no pycnidia. If the chemicals of this formula be diluted 50 times, the organism will fruit abundantly upon it. This organism was found to be greatly overfed by the ordinary laboratory media, and under the influence of the great excess of food grew and grew until the by-products of metabolism checked growth or destroyed the organism.

The differences in media were not so much in the food which they contained—for an examination of published analyses will show all necessary elements for growth and reproduction in almost any plant—as in the acid or alkaline reaction which the medium gave when prepared, the reaction maintained, and the concentration or relative scantiness of carbohydrate

and protein. The least adapted synthetic solution for this fungus (Raulin, 1869), could be made to yield pycnidia by the addition of lime, which probably counteracted the acidity; and pea cultures in which the mycelium was submerged and nearly dead could be made to grow and produce pycnidia by mere acidification. Furthermore, pea cultures to which sugar is added to balance the protein produce abundant pycnidia in the aerial hyphæ.

A consideration of the various laboratory media shows them to be rather purposeless, clumsy devices, in which this organism is overfed. Except the very simplest ones, none have warrant for existence if considered from the point of view of adaptability for a specific purpose. The great similarity of results on the various media seems to require the conclusion that these foodstuffs are not specific. Any fruit or vegetable is a full nutrient for almost any organism if the material be made properly soluble, and any harmful acid or alkaline reaction or otherwise unfavorable concentration be adjusted. Probably any biological product can likewise be utilized. Our methods have made a fetish of variety and have completely neglected the contributing factors.

As has been said, fungi behave alike in their relations to the substrata in the vast majority of cases. That the findings for this organism apply to others seems entirely probable. In many ways confirmatory evidence is to be found in the present practices. A certain medium is discovered which gives fruiting bodies for some fungus. A number of other organisms not at all related, in spite of differences in life relation, are also found to fruit upon this medium.

A consideration of one of the best preparations devised for fruit-body formation is very interesting. Shear's corn-meal agar is made by stiffening with agar the infusion obtained from four teaspoonfuls of corn meal (Shear and Wood, 1913). This medium is suitable for fructification for this organism, because it gives a scanty food supply, yet sufficient readily available to produce the growth necessary for pycnidium formation. The ratio of carbohydrate to protein is such that the reaction remains acid. Reasoning from such similar phenomena, a rather general application may be made. Any organism of this type can be made to grow and fruit upon a synthetic substratum containing the essential components, provided that the ratio of the components, hence the acid or alkaline reaction, and the concentration, be adjusted to the limits demanded by the particular organism. This assumes that the factors of light, temperature, aeration, etc., also fall within their own suitable limits.

We have, therefore, within the reach of experimental work the possibility of developing an environment which can be so defined that it can always be duplicated, suitable for a great group of organisms (Thom, 1910). With such a chemically and physically defined environment the classification of organisms could be placed upon a sounder working basis.

It is commonly admitted that the description of an organism must be taken under the assumption of some definite environment. The great mass of media in common use, the uncertainty of composition, the lack of standardization, and the usual failure to bring about fructification have left the description of fungi with only the natural habitat as a fixed environment. With forms of comparatively simple morphology this standard has led to the classification by hosts, with its attendant multiplicity of species. A firm basis for taxonomy can be arrived at, and simplification can come, only from a standardized environment.

As has been indicated in the preceding discussion, the physical environment must also be defined. With the growth of our knowledge of the forms we shall be able to a great extent to analyze the complex of forces. In the present paper one such force has been emphasized and its action discovered to be related to the liberation of energy by oxidation.

Light was found to be essential for reproduction. If light be absent or insufficient, although all other requirements were satisfied—with a medium suitable for growth and food supply, aeration, acid reaction, temperature, all within the proper limits—pycnidium production will not take place. Instead, aerial mycelium is formed, and eventually the organism goes into a static condition. The light factor, as others, has its limits. Weak light will not allow pycnidium production. This factor differs from the others in that its action need not be continuous. It is therefore of direct stimulative nature. A short exposure to strong diffuse light of cultures from dark conditions, which are otherwise ready for pycnidia formation, gives the necessary stimulus during a further period in the dark. When the effect of the stimulation is spent in the production of a few pycnidia, a second exposure is necessary for a second inauguration of the process.

The action of light in thus unlocking these forces is very satisfactorily explained by the experiment in which a few drops of hydrogen peroxid were used to replace the light stimulus. Other oxidizing agents also serve to stimulate fruit-body formation. The protoplasm of well-nourished mycelium is rich in oily reserve materials, and the action of light may oxidize these bodies and change them from emulsions of poor mobility to materials of great diffusibility. Accompanying this we have a releasing of energy, and fruit-body formation is inaugurated. The mechanism of this process is not known at all, but Herzog (1903) has shown that the sporulation of yeast is affected by temperature, and the curve for the variation in amount produced by temperature is a typical enzym curve.

Hydrogen peroxid added to a pea-broth culture, to a rich sugar solution, or to a young growing culture on corn broth does not immediately lead to fruit-body formation, nor does the action of light on such cultures lead to it. The action of light is modified and controlled by the condi-

tion of the mycelium, and this we have seen is a resultant of the environmental factors. In other words, we must consider in what way a mass of mycelium with checked vegetative growth is influenced to reproduction, while one in active growth is unaffected.

The cause of this relation to light, or, better, to oxidation, is understood if we take into account the fact that among organisms and among parts of the same organism there exists a strong competition for oxygen. In the cell itself the various processes inhibit and influence each other by their oxygen relations. Oxidation is never at its maximum in the cell under ordinary conditions, as simple tests with increased oxygen tensions show (Porodko, 1904). Organisms well aerated grow better than those in an air supply below the optimum. The action of oxidation is to release energy. The materials oxidized are either the foodstuffs suitable for nutrition or the cell material which growth has stored up. Euler (1909) contrasts growth, a stretching process, with reproduction, a differentiating and formative process. Growth is a process which is gradual, and it takes place even if only a small amount of energy be available. It is a process taking less energy than reproduction, as all respiration experiments have shown. The great consumption of energy in reproduction is doubtless associated with the great amount of nuclear protoplasm which must be formed. Growth, therefore, is the process first inaugurated, and the one which continues so long as the food supply is abundant and outer conditions permit. It is a static condition, as reproduction is dynamic.

In the hunger state the oxidations are different to a marked degree, as Kosinski (1901) has discovered, and here we have the cell reserve gradually drawn upon. The fats and even the proteins may be oxidized, according to Purievich (1900). But in this hunger state the respiration is reduced, according to Kosinski; hence, the working is slow. These metabolic relations, in spite of their great complexity, balance each other.

It would seem that reproduction is not possible under conditions favoring growth, because the oxygen supply is all used in ordinary metabolism. With the hunger state, respiration is reduced. Oxidation becomes vigorous if it be stimulated by light. No doubt any catalytic agent would be similarly effective. Once in this hunger state, oxidation, if augmented, takes place upon the rich cell stuffs, with the liberation of much energy. This energy is used in reshaping the reserve stuffs into complex protein bodies—the spores. The sharper the hunger condition is made, the more striking the reaction in pycnidium production. The sudden withdrawal of the food supply by the transfer of richly-growing mycelium to lower concentrations or to distilled water, checks ordinary assimilation, with its attendant use of oxygen. If oxidation of the cell reserves be inaugurated by light or some strong oxidizing agent, fructification takes place.

We may now consider other factors in the light of this theory. Experiment has shown that aeration is essential for reproduction. The action of light upon the protoplasm is dependent upon the oxygen supply. Aeration may work to continue the oxidizing process by the removal of end products, thus allowing oxidations to proceed to completion. In many cases recorded in the literature the effect of transpiration is to further the exchange of gases. The action of low temperature was to check growth, and pycnidium production was found to start. Euler (1909) states that lowering the temperature affects the oxidation process to a lesser degree than it affects other processes.

The action of the acid reaction is interesting and confirmatory. So far in this discussion the mechanics of the oxidation have not been considered. Oxidations in plants are generally believed to take place through the activity of oxidases of various sorts. As is well known, light activates this type of enzym, although it is detrimental to such enzyms as diastase (Euler, 1909, p. 97). The pronounced and sudden blackening of cultures about to produce pycnidia is very significant and can be best explained by the oxidation of some leuco compound by an oxidase (Kruse, 1910, p. 787). Some oxidases are known which work better in a slightly acid medium. We have seen that for this organism an alkaline medium was prejudicial to reproduction. The effect of acid reaction in favoring the reproductive process has not been explained, but it may have some connection with the enzymotic process. At any rate, an oxidation of oily stuffs to fatty acids would give a medium suitable for further activity of these ferments.

The formation of pycnidia in the aerial mycelium and in fact the whole series of complex reactions which Klebs (1900) has associated with "*Luftleben*" become much more comprehensible if we view them from the point of view of oxidation.

The replacement of the light factor by hydrogen peroxid thus becomes of great importance in reducing to simple terms the phenomena encountered. Light can unlock in suitable mycelium the reproductive process. This it does by its catalytic influence. The action may be due to the activation of oxidases along with the inauguration of a reaction (acid) favorable to their continued action; but this oxidation thus set up does not proceed to reproduction if the growth process is consuming the energy. If growth is not able to proceed, owing to scanty food supply or some checking influence, then the catalytic action of light inaugurates a building of the stored foodstuffs into complex fruiting bodies.

This general discussion may now be summarized. In the historical portion of the paper it was seen that the environment may be viewed as a directive and collective force which can be utilized for unfolding the life history of an organism. The great generalizations of Klebs are broad, and by their very broadness make possible acceptance in a wide

range of cases. Their teachings can not, however, be made the basis for research without the development of methods of attack suitable to a series of forms. The method of this paper may be used for similar organisms.

The first part of the paper may be interpreted as a determination of the limits of the life processes, which, when once determined, allow in the latter part of the paper a manipulation of them. The knowledge of the factors and their optima made possible a development of an environment especially fitted for growth and reproduction.

The proposition of Klebs, that the limits of reproduction are narrower than those of growth, is fully substantiated. Klebs further pointed out that growth and reproduction are processes opposed to each other. This is true for the organism studied.

The action of light has led to an insight into the mechanism of this opposed action. It has shown that growth, the static condition, is opposed to reproduction, a dynamic condition. Where one process is storing energy, the other is a process consuming energy. The equilibrium within the cells needs to be upset by some oxidizing force in the case of this fungus to inaugurate fruit-body formation in susceptible mycelium.

It is not concluded from the experiments with this species that light is a specific factor which will cause reproduction to take place in all forms, once growth is checked, although it may be expected to be an important condition in related organisms. But, in view of the great similarity of behavior in all the forms tested so far with respect to growth and reproduction, it may be concluded that in them some stimulus becomes operative when an organism is in the hunger state which starts the utilization by oxidation of the stored food supply and leads to the phenomenon of reproduction.

SUMMARY

This paper gives the results of experiments performed with *Plenodomus fuscomaculans*, a fungus pathogenic to the apple. The specific problem undertaken was the determination of the effects of various controlled environmental factors upon the growth and reproduction of this fungus.

The historical development of the art of culturing organisms has been traced from the first crude cultures to the present elaborate technic. The simultaneous development of our knowledge of the physiology of organisms has been briefly summarized. This survey shows that the environmental factors may greatly influence the life processes of organisms. Organisms have been cultured in the laboratory in an imitative or haphazard way, with a chance of finding a suitable environment. Owing to the great variety of available methods and the great plasticity of organisms, this course has been productive of results with some forms. Another type of research has sought to find the relation of the organism

to its environment and by manipulation of the environmental factors to discover the various phases of life history. Although many related forms have been grown in pure culture, very little physiological work of this type has been done with the Sphaeropsidales.

The organism was found to have a wider range of conditions suitable for growth than for reproduction. The base level of conditions necessary for growth is found in conductivity water at low temperatures. Reproduction requires more favorable conditions. Pycnidium production took place only in cultures exposed to light. The ordinary room temperatures were sufficient. Abundant aeration is essential. Transpiration is a factor of secondary importance. A slight acid reaction, especially at the close of the growing period, is a necessary condition. The value of a medium depends largely upon the acid or alkaline reactions present, not alone at the beginning but at the close of the growing period. Autointoxication was observed and was traced to excess of either acid or ammonia, which was the product of too great a proportion of either carbohydrate or protein, respectively.

As has been said, the quantity of foodstuff necessary for growth is extremely minute. Pycnidium production requires more food, but the meager amount present in distilled water is sufficient to allow the production of a few pycnidia. On the other hand, the fungus is able to tolerate very rich food supplies, but pycnidium production in solutions is restricted to *M*/100 or perhaps *M*/50 sugar concentration. Exact limits are hard to determine, because of the formation of mats or films in solutions, which effectively wall off much of the food supply. Fructification in the case of rich media takes place in the aerial hyphæ, and no doubt this relation corresponds with the conditions in solutions.

Magnesium sulphate and potassium dihydrogen phosphate in very dilute solutions furnish the necessary mineral elements for growth and reproduction. The carbon supply may be taken from a wide range of compounds of alcoholic structure. The carbohydrates furnish food materials in most available form, and, of these, xylose and maltose produce the best growth. The carbohydrates do not seem to be specific in producing fruiting bodies, and almost any are suitable if taken at the right dilution. The nitrogen assimilation is greatly influenced by the type of carbon nutrition.

The minerals mentioned and maltose and asparagin at the ratio of 5 to 1 seem to offer the most favorable combination, although others are suitable. From the experiments a medium was selected which though of entirely known composition gave better growth than any other tried. This synthetic solution had a scant amount of food supply, yet enough to permit a quick, vigorous growth. It retains the acid reaction till the close of the growing period. A study of this medium gave a basis for a criticism of results obtained with the common laboratory combinations.

The problem of this paper was a study of the effect of environmental factors upon this organism, especially as they influenced growth and reproduction. The experiments here reported verify the conclusions of Klebs and extend them for an untested group of organisms, the Sphaeropsidales. As has been pointed out, in this paper the method of approach was different from the inductive methods used by Klebs in drawing his conclusions, since the methods employed here were deductive, based on our knowledge of the reactions of other organisms. The experiments with *Plenodomus fuscomaculans* give a method applicable to related forms. The results of this physiological work give a basis for practical recommendations as to the culture of other organisms, as well as evidence of the feasibility of developing a standard synthetic solution which would make possible a standardization of environments for diagnostic purposes.

The action of light, when pushed to a last analysis and when considered in view of the experiment in which hydrogen peroxid and other oxidizing agents replaced it, is seen to be of either an oxidizing or a catalytic type. This led to the development of a theory to explain the mechanism of the opposed action of growth and reproduction. This theory sees in the competition for oxygen the fundamental reason for the absence of fructification under conditions which allow abundant growth.

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EFFECT OF ELEMENTAL SULPHUR AND OF CALCIUM SULPHATE ON CERTAIN OF THE HIGHER AND LOWER FORMS OF PLANT LIFE [1]

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INTRODUCTION

A study of the literature [2] shows that a number of investigators have noted a beneficial effect when elemental sulphur or sulphates are added to certain soils. The number of these investigations and also the types of soil and plants employed are limited. Certain workers report no beneficial effects from the addition of sulphur or sulphates to soil, and in isolated cases an injurious effect has been noted. Just how the sulphur or its compounds act is little understood, but there are two plausible explanations: (1) That it acts as a fertilizer, supplying the sulphur needed for plant growth, and (2) that it acts as a corrective agent—i. e., it favors beneficial groups of bacteria, while injurious forms are retarded in growth. However, the problem of sulphur and sulphates in agriculture is still far from being solved. This is especially true in the case of the effect of sulphur and sulphur compounds upon micro-organisms. In order to study this phase of the problem, a series of experiments was planned.

PLAN OF WORK

The object of these experiments was (1) to note the effect of sulphur and sulphates upon the soil micro-organisms and on pure cultures of legume bacteria, and (2) to note the effect of sulphur and sulphates upon the growth of red clover (*Trifolium pratense*).

For the experiments with mixed cultures, fresh soil was used as an inoculum. For legume bacteria all materials were sterilized, and the nutrient medium was inoculated with a pure culture of bacteria from the nodules of red clover.

[1] Paper from the Laboratories of Agricultural Bacteriology and Agricultural Chemistry of the University of Wisconsin.

[2] Hart, E. B., and Tottingham, W. E. The relation of sulphur compounds to plant nutrition. *In* Jour. Agr. Research, v. 5, no. 6, pp. 233–250. 1915. Literature cited, p. 249.

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EFFECT OF ELEMENTAL SULPHUR AND SULPHATES ON SOIL BACTERIA

MIXED CULTURES

For these experiments ten 1-gallon jars containing 2 kgm. each of Miami silt loam taken from the Wisconsin Experiment Station farm were used. The analysis of this soil is as follows:

	Per cent.
Potassium	2.16
Nitrogen	.15
Phosphorus	.15
Sulphur	.016
Calcium carbonate	.33
Humus	1.38

The moisture content of the soil was held at 18 per cent, or about half-saturation. Each jar was covered with a layer of cotton and gauze to prevent contamination, and was incubated at 28° C. Various amounts of sulphur and of calcium sulphate were added to the pots, as shown in Table I. At definite intervals samples were taken from the jars and bacterial counts as well as determinations of ammonia and of nitrates made. The results of the latter are given in Table I.

TABLE I.—*Effect of calcium sulphate and elemental sulphur on soil bacteria*

Treatment.	Number of organisms per gram of soil after—				
	12 days.	30 days.	44 days.	72 days.	93 days.
Untreated	6,866,000	8,746,000	10,790,000	6,350,000	10,382,000
Given 0.01 per cent of calcium sulphate	6,866,000	10,544,000	13,900,000	6,590,000	11,026,000
.05 per cent of calcium sulphate	8,506,000	14,140,000	13,789,000	8,028,000	9,945,000
.10 per cent of calcium sulphate	7,221,000	7,923,000	13,060,000	7,923,000	9,624,000
.50 per cent of calcium sulphate	8,290,000	8,029,000	13,420,000	7,548,000	10,305,000
1.00 per cent of calcium sulphate	8,580,000	9,585,000	12,938,000	7,668,000	9,945,000
Untreated	6,590,000	9,166,000	8,626,000	6,949,000	9,705,000
Given 0.01 per cent of sulphur	7,429,000	8,746,000	8,866,000	7,923,000	8,626,000
.05 per cent of sulphur	9,106,000	8,866,000	0,065,000	7,668,000	8,116,000
.10 per cent of sulphur	8,290,000	8,208,000	10,300,000	6,590,000	8,866,000
.50 per cent of sulphur	8,864,000	11,020,000	4,914,000	3,594,000	2,896,000
1.00 per cent of sulphur	6,504,000	7,070,000	2,635,000	2,329,000	719,000

The data show that calcium sulphate in the quantities used apparently has little effect on the number of soil organisms. Elemental sulphur, however, decreases the number of soil organisms that grow on agar plates. This decrease is not noticed until after 44 days, and only in soils to which 0.05 and 1 per cent of sulphur had been added. Quantitative acidity tests of the soils of these two jars showed it to be distinctly acid. This is corroborated by the work of Lint,[1] who has shown that in soil elemental sulphur is oxidized to sulphate and that the acidity produced is proportional to the amount of sulphur added. Acidity determinations were made according to Truog's[2] method and are given in Table II.

[1] Lint, H. C. The influence of sulphur on soil acidity. *In* Jour. Indus. and Engin. Chem., v. 6, no. 9, p. 747–748. 1914.

[2] Truog, E. A new test for soil acidity. Wis. Agr. Exp. Sta. Bul. 249, 16 p., 3 fig., 1 pl. 1915.

TABLE II.—*Acidity of soil treated with elemental sulphur*

Treatment.	Calcium oxid necessary to neutralize acid in 10 gm. of soil.
	Gm.
Untreated	0. 0000
Given 0.01 per cent of sulphur	. 0000
.05 per cent of sulphur	. 0000
.10 per cent of sulphur	. 0011
.50 per cent of sulphur	. 0369
1.00 per cent of sulphur	. 0668

The results of these determinations show that the acidity produced by the oxidation of elemental sulphur to sulphate is proportional to the amount of sulphur added. In the samples to which 0.01 and 0.05 per cent of sulphur had been added, the soil contained enough lime to neutralize the acidity.

Change in reaction is probably the cause of the decrease in the number of the soil organisms. Abundant mold growth was found on the surface of the acid soils.

Table III shows that calcium sulphate in the quantities used has no effect on the production of ammonia in the soil. Elemental sulphur, however, in concentrations of 0.5 and 1 per cent increases the production of ammonia to a marked degree. This increase is noticeable after 44 days.

TABLE III.—*Effect of calcium sulphate and elemental sulphur on the production of ammonia in the soil*

Treatment.	Quantity (in milligrams) of ammonia nitrogen per 100 gm. of soil after—				
	12 days.	30 days.	44 days.	72 days.	93 days.
Untreated	3.99	3.19	3.40	3.21	3.23
Given 0.01 per cent of calcium sulphate	3.19	2.38	3.32	2.80	3.48
.05 per cent of calcium sulphate	3.19	2.21	3.06	3.06	3.57
.10 per cent of calcium sulphate	3.82	2.38	3.23	3.32	3.23
.50 per cent of calcium sulphate	3.19	2.29	3.36	3.40	3.57
1.00 per cent of calcium sulphate	3.82	2.21	3.06	3.23	3.40
Untreated	3.97	3.19	3.12	2.97	3.40
Given 0.01 per cent of sulphur	3.91	2.38	3.19	2.72	3.23
.05 per cent of sulphur	3.19	2.29	3.06	2.46	3.23
.10 per cent of sulphur	3.06	2.21	3.23	2.55	3.16
.50 per cent of sulphur	3.82	2.89	5.95	7.31	8.26
1.00 per cent of sulphur	3.95	2.89	6.80	7.31	9.52

The data in Table IV show that calcium sulphate in the quantities used does not materially affect the formation of nitrates in the soil. Elemental sulphur, on the other hand, in concentrations of 0.5 and 1 per cent decreases nitrate formation. This decrease is noticeable after 30 days. Previous to this time the sulphur does not seem to injure nitrate formation. Concentrations of sulphur lower than 0.5 per cent have no

appreciable effect on nitrification. It should be noted that while the bacterial counts begin to decrease after 44 days, the ammonia content begins to increase at this time.

TABLE IV.—*Effect of calcium sulphate and elemental sulphur on nitrate production in the soil*

Treatment.	Quantity (in milligrams) of ammonia nitrogen per 100 gm. of soil after—				
	12 days.	30 days.	44 days.	72 days.	93 days.
Untreated	1.87	1.34	2.95	3.93	4.70
Given 0.01 per cent of calcium sulphate	2.12	1.25	2.35	4.13	5.05
.05 per cent of calcium sulphate	2.42	1.01	2.49	4.58	5.72
.10 per cent of calcium sulphate	1.14	1.25	2.10	4.03	5.41
.50 per cent of calcium sulphate	1.86	1.23	2.45	2.92	4.99
1.00 per cent of calcium sulphate	1.35	1.82	2.39	2.87	4.51
Untreated	1.53	1.66	2.93	3.93	4.35
Given 0.01 per cent of sulphur	1.80	1.99	2.65	3.13	4.13
.05 per cent of sulphur	2.17	1.88	2.29	3.20	4.53
.10 per cent of sulphur	1.38	1.69	2.92	4.13	4.93
.50 per cent of sulphur	1.24	.54	1.41	1.14	.19
1.00 per cent of sulphur	.94	.54	.64	.95	.85

PURE CULTURES

In order to determine the effect of calcium sulphate on pure cultures of legume bacteria (red clover), Ashby's solution, minus the sulphate, was used. To 100 c. c. portions of this solution in 10 large Erlenmeyer flasks were added 30 gm. of pure quartz sand and various amounts of calcium sulphate. The sand was used to aid in breaking up the aggregates of bacteria when samples were taken for counts. All cultures were incubated at 20° C., and at intervals of one and two weeks bacterial counts were made. The results of these counts are given in Table V.

TABLE V.—*Effect of calcium sulphate on the growth of red clover organisms in Ashby's solution*

Treatment.	Number of organisms per cubic centimeter of solution after—		
	0 day.	7 days.	14 days.
Untreated	30,000	53,000,000	157,000,000
Given 0.01 per cent of calcium sulphate	30,000	139,000,000	425,000,000
.02 per cent of calcium sulphate	30,000	177,000,000	400,000,000
.05 per cent of calcium sulphate	30,000	198,000,000	450,000,000
.10 per cent of calcium sulphate	30,000	121,000,000	350,000,000

The data show that the numbers of bacteria that grow on Ashby's agar were increased by the addition of calcium sulphate. The increase is very marked after both 7 and 14 days. It should be noted that 0.01 per cent of calcium sulphate is apparently just as efficient in producing an increase in the number of bacteria as is 0.1 per cent. This seems to indicate that only a trace of calcium sulphate is needed to stimulate the legume bacteria.

This experiment was repeated, using soil solution in place of Ashby's solution. For this purpose 1 kgm. of Miami silt loam was placed in a large container, 1 liter of distilled water added, and the entire mass boiled for one hour. It was next filtered, and 0.05 gm. of dipotassium phosphate and 1 gm. of mannite were added. This was then put into ten 500 c. c. flasks and 30 gm. of quartz sand added. Various amounts of calcium sulphate were used. The flasks were sterilized, and when cool were inoculated with a pure culture of red-clover bacteria. All cultures were incubated at 23° C. At intervals of one, two, and three weeks bacterial counts were made. These results are given in Table VI.

TABLE VI.—*Effect of calcium sulphate on the growth of red-clover organisms in soil solution*

Treatment.	Number of organisms per cubic centimeter of solution after—			
	0 day.	7 days.	14 days.	36 days.
Untreated	180,000	63,000,000	145,000,000	146,000,000
Given 0.01 per cent of calcium sulphate	180,000	135,000,000	176,000,000	237,000,000
.02 per cent of calcium sulphate	180,000	125,000,000	178,000,000	244,000,000
.05 per cent of calcium sulphate	180,000	125,000,000	269,000,000	259,000,000
.10 per cent of calcium sulphate	180,000	138,000,000	185,500,000	262,000,000

From the data it is evident that the addition of calcium sulphate stimulates the growth of red-clover organisms in pure cultures to the extent of more than 100 per cent. The results of this test agree with those obtained in Ashby's solution—i. e., that small amounts of calcium sulphate are apparently as beneficial as larger amounts.

EFFECT OF SULPHUR AND SULPHATES ON HIGHER PLANTS IN ARTIFICIAL MEDIA

Various experiments were made with the view of determining the effect of calcium sulphate and sulphur upon the growth of clover and upon nodule formation. This was tested first in artificial media. The medium consisted of a soft synthetic agar prepared from 1 liter of tap water, 5 gm. of dipotassium phosphate, and 7 gm. of agar. This medium was sufficiently firm to support the seeds. Thirty c. c. of the melted agar plus various quantities of calcium sulphate were added to each of 50 test tubes. In order to reduce the individual variation between the plants, 10 parallel tubes were used. The tubes were sterilized, and then two seeds of red clover were planted in each. After inoculation the cultures were removed to the greenhouse. At the end of two weeks greater root development was noted in the calcium-sulphate test tubes than in the untreated ones. In the older plants the increase in root development became most marked. The tops, however, failed to show any difference in size. In the tubes to which 0.1 per cent of calcium sulphate had been added, the plants were slightly smaller than the

others. At the end of six weeks the plants were removed and the roots measured. There was a distinct difference in root development, as shown in Table VII. Plate LVI, figure 1, shows very plainly the decided differences in root development. The results indicate that the increase in root development is as great with only 0.01 per cent of calcium sulphate added as with larger amounts. The test tubes treated with calcium sulphate were chosen at random from the calcium-sulphate series. They appear lighter because of the suspension of small particles of the salt in the agar.

The results of this experiment show that calcium sulphate greatly increases root development. However, in concentrations as high as 0.1 per cent, growth is slightly retarded. The increase in root development may be of considerable importance, first, because it enables the plant to reach out over a greater area for nourishment, and second, because of the greater field, the plant will be able to withstand drought better and thrive on poorer soil. The increase in root development may be the cause of the increase in the yield of clover when calcium sulphate is added to the soil. This is in confirmation of the work of Hart and Tottingham.[1]

These results are given in Table VII, which represents the average of 10 test tubes for each concentration used.

TABLE VII.—*Effect of calcium sulphate on the growth of red clover*

Treatment.	Length of root.	Length of stem.
	Cm.	*Cm.*
Untreated	3.8	4.2
Given 0.01 per cent of calcium sulphate	5.1	4.19
.02 per cent of calcium sulphate	5.5	4.7
.05 per cent of calcium sulphate	5.01	4.6
.10 per cent of calcium sulphate	4.93	3.3

EFFECT OF SULPHUR AND CALCIUM SULPHATE UPON CLOVER GROWN IN VARIOUS TYPES OF SOILS

The effect of calcium sulphate upon clover grown on Miami silt-loam soil was tested. For this experiment ten 1-gallon jars were used. Four kgm. of Miami silt-loam soil and various amounts of calcium sulphate were added to each. The jars were kept in the greenhouse and the moisture content held at 18 per cent. Each jar was seeded with red clover and then inoculated with a pure culture of red-clover organisms. After two weeks the jars were thinned to 10 plants.

During the first few weeks there was no apparent difference in the size of the plants. At the end of seven weeks an increase in growth in jars 3 to 8, inclusive, was noted. In jars 9 and 10, to which 0.1 per cent of calcium sulphate had been added, there was a decrease in growth. Four

[1] Hart, E. B., and Tottingham, W. E. Op. cit.

representative plants were removed from each jar. The roots of the plants grown in the sulphate-treated soil were longer and more branched than those of the plants grown in the untreated soil. There was an apparent increase in the number of nodules grown in the sulphate-treated series, except in the case of plants grown on soil to which 0.1 per cent of calcium sulphate had been added. The number of nodules on the above plants were about the same as on the plants grown in untreated soil. It must be remembered that the plants grown in the soil containing 0.1 per cent of calcium sulphate were smaller and therefore would naturally contain fewer nodules than the larger plants. Plate I,VI, figure 2, illustrates these effects. The plants in group A were taken from the untreated soil; B, from the soil to which 0.01 per cent of calcium sulphate had been added; C, from soil to which 0.02 per cent had been added; D, from soil to which 0.05 per cent had been added; and E, from soil to which 0.1 per cent of calcium sulphate had been added. Note the marked increase in root development in B, C, D, and even E, where the plants are the same size as those in group A; also note that group E, to which 0.1 per cent of calcium sulphate was added, and D, to which 0.05 per cent was added, show no greater growth than A, the untreated, while groups B and C show an increase in top as well as root. The illustration shows very distinctly the increase in length of root and also the decrease in the growth of the plant under high concentrations of calcium sulphate. It is apparent that the addition of 0.02 and 0.05 per cent of calcium sulphate gave the most beneficial results.

TABLE VIII.—*Effect of calcium sulphate on the growth of red clover in soil*

Treatment.	Number of nodules.	Average of group.	Length of root.	Average of group.
	Cm.	*Cm.*	*Cm.*	*Cm.*
Untreated	9		6.5	
Do	12	10	8.5	7.2
Do	8		6.8	
Do	10		7.0	
Given 0.01 per cent of calcium sulphate	29		8.0	
Do	33	31	10.0	9.6
Do	11		10.5	
Given 0.02 per cent of calcium sulphate	51		9.0	
Do	34		8.0	
Do	17	33	9.6	9.5
Do	48		12.0	
Given 0.05 per cent of calcium sulphate	32		8.5	
Do	45		9.0	
Do	36	29	6.5	9.2
Do	18		11.3	
Given 0.1 per cent of calcium sulphate	19		11.5	
Do	13		7.0	
Do	11	14	7.5	7.6
Do	12		8.5	
Do	11		7.5	

The data in Table VIII show that calcium sulphate does increase the growth of the clover within a certain concentration. In amounts between 0.02 and 0.05 per cent it appears to be most beneficial. The results also show that calcium sulphate increases the root development and the number of nodules.

The effect of calcium sulphate on clover grown on Sparta acid sand was tested. Six kgm. of the sand admixed with 1 gm. of dipotassium phosphate were placed in each of ten 1-gallon jars. The composition of the Sparta acid sand used was as follows:

	Per cent.
Potassium	1.16
Nitrogen	.062
Phosphorus	.034
Organic matter	1.51

The jars were kept in the greenhouse and the moisture content held at 18 per cent. Each jar was seeded to red clover and then inoculated with a pure culture of red-clover organisms. After two weeks the jars were thinned to 20 plants in each. The plants grew luxuriantly, but there was no apparent difference in size until the sixth week. In jars 7 and 8, to which 0.05 per cent of calcium sulphate had been added, the increase in growth was considerable, while in jars 9 and 10, to which 0.1 per cent of calcium sulphate had been added, there was no appreciable increase. The jars to which 0.01 and 0.02 per cent of calcium sulphate had been added showed an increase in growth, but this increase was less than in jars 7 and 8. The green and dry weights of the clover were taken. The average weights of the clover are given in Table IX.

TABLE IX.—*Effect of calcium sulphate on red clover grown in Sparta acid sand*

Treatment.	Weight of crop.	
	Green.	Dry.
	Gm.	*Gm.*
Untreated	110.6	19.4
Given .01 per cent of calcium sulphate	131.1	21.2
.02 per cent of calcium sulphate	146.7	23.7
.05 per cent of calcium sulphate	168.5	24.6
.10 per cent of calcium sulphate	145.8	17.5

These results show that calcium sulphate increases the growth of clover grown on Sparta acid sand. The increase, however, is confined to certain concentrations. The greatest increase was obtained at concentrations of 0.02 and 0.05 per cent.

EFFECT OF ELEMENTAL SULPHUR ON GROWTH OF RED CLOVER

For this experiment ten 1-gallon jars, each containing 6 kgm. of Miami silt-loam soil, were used. Various amounts of sulphur were added. The jars were kept in the greenhouse and the moisture content

held at 18 per cent. After four weeks these were seeded with red clover and inoculated with a pure culture of red-clover organisms. Two weeks later the number of plants was reduced to six per jar. There was no appreciable difference in the size of the plants until the fourth month. At this time those in the sulphur series showed an increase in growth. At the end of the fifth month this increase was more marked. The leaves of the plants in the jars to which 0.05 per cent of sulphur had been added were tinged with red at the edges. The stem also showed this red coloration, but to a lesser degree. At the end of the fifth month the tops were cut and weighed, green and dry, with the results shown in Table X.

TABLE X.—*Effect of elemental sulphur on the growth of red clover*

Treatment.	Weight of crop.	
	Green.	Dry.
	Gm.	*Gm.*
Untreated	25.3	6.25
Given .01 per cent of sulphur	32.6	6.90
.02 per cent of sulphur	29.4	6.75
.05 per cent of sulphur	30.8	6.80
.10 per cent of sulphur	34.0	7.00

The sulphur series showed a slight increase in yield. Several of the plants died, so that the number of plants in the various jars varied. The results therefore are not final. It seems safe, however, to say that sulphur increased slightly the yield of clover in Miami silt-loam soil. After the tops were cut the roots were carefully removed and washed. There was no apparent difference in the size or the number of nodules in the treated and the untreated series. All of the roots contained a great number of nodules.

SUMMARY

(1) Calcium sulphate, when added to a soil, apparently has no marked effect on the total number of bacteria that grow on agar plates; nor does it produce any marked increase in ammonification or nitrification. This confirms the observations of Fred and Hart.[1]

(2) Large amounts of elemental sulphur cause a decrease in the total number of bacteria that grow on agar plates, but produce an increase in ammonification at concentrations of 0.05 per cent. This increase in ammonia is accompanied by a parallel decrease in nitrate formation. The decrease is very probably due to the acidity or toxicity produced by the oxidation of sulphur.

[1] Fred, E. B., and Hart, E. B. The comparative effect of phosphates and sulphates on soil bacteria. Wis. Agr. Exp. Sta. Research Bul. 35, p. 35–66, 6 fig. 1915.

(3) Calcium sulphate stimulates the growth of pure cultures of red-clover bacteria in nutrient solutions and in soil extract. The increase is as great with 0.01 per cent as with 0.1 per cent.

(4) The root development of red clover is increased by calcium sulphate, 0.01 per cent being apparently as efficient in producing this increase as 0.1 per cent.

(5) In small amounts calcium sulphate increases the yield of red clover and also the number of nodules. Concentration as high as 0.05 to 1 per cent, however, produces no increase in growth.

(6) The application of elemental sulphur to Miami silt-loam soil increased but slightly the yield of clover and apparently did not affect root development or nodule formation. In producing this slightly increased growth 0.01 per cent was as efficient as were higher concentrations.

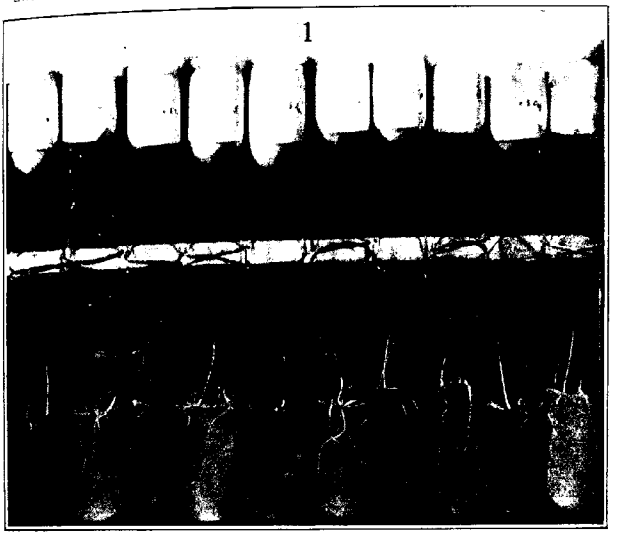
(7) A review of the results of these experiments shows that calcium sulphate in soil does not produce any marked effect on the bacteria commonly found on agar plates, but does increase the growth of the legume bacteria. It also increases the yield of red clover, which is accompanied by a greater root development and a greater number of nodules.

(8) The addition of sulphur increases the ammonification, but decreases nitrification and the total number of soil organisms. It increases the yield of red clover but slightly and does not affect the root development nor the number of nodules.

PLATE LVI

Fig. 1.—Red-clover plants, showing the effect of treatment with calcium sulphate. The plants in these test tubes show the contrast in size of root between the treated and untreated tubes. The treated tubes were selected from various concentrations. Beginning at the left, tubes 1, 3, 5, 7, and 9 are untreated; tubes 2, 4, 6, 8, and 10 are of the calcium-sulphate series. Note the decided increase in length of root of the plants in the treated tubes as compared with those in the untreated.

Fig. 2.—Group *A*, untreated; *B*, 0.1 per cent of calcium sulphate added to Miami silt-loam soil; *C*, 0.02 per cent added; *D*, 0.05 per cent added; *E*, 0.1 per cent added.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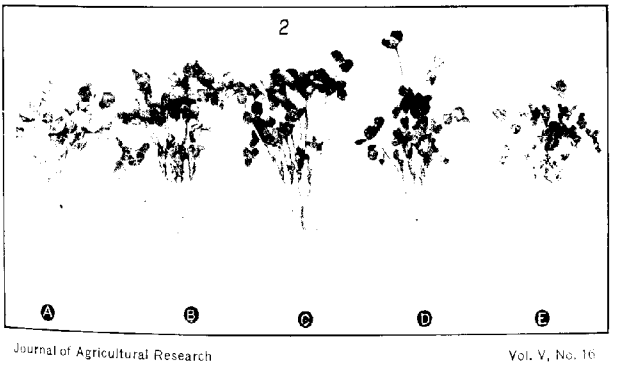
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